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(71) Applicant *(for all designated States except US)*: STERLING WINTHROP, INC. [US/US]; 9 Great Valley Parkway, Malvern, PA 19355 (US).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: SNOW, Robert, A. [CA/US]; 118 Cratin Lane, West Chester, PA 19380 (US). KRUSE, Lawrence, I. [US/US]; 646 Clinton Avenue, Haddonfield, NJ 08033 (US). BLACK, Christopher, D., V. [GB/US]; 2815 Aquarius Avenue, Silver Spring, MD 20906 (US). SHEARMAN, Clyde, W. [US/US]; 607 Chesterville Way, West Chester, PA 19382 (US).

(74) Agents: KATZ, Martin, L.; Dressler, Goldsmith, Shore & Milnamow, Ltd., Two Prudential Plaza, Suite 4700, Chicago, IL 60601 (US) et al.

(81) Designated States: AU, CA, JP, US, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

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(54) Title: IMMUNOREACTIVE REAGENTS EMPLOYING DIHYDROFOLATE REDUCTASE

(57) Abstract

This invention describes a non-radioactive targeting immunoreagent comprised of the residue of a proteinaceous active site of a dihydrofolate reductase enzyme (DHFR), a linking group, and the residue of an immunoreactive material together with a radioactive delivery agent comprised of the residue of a ligand which has an affinity for non-covalent binding to said DHFR receptor moiety, a linking group, and the residue of a radioactive agent. This invention also describes a non-radioactive targeting immunoreagent comprised of the residue of a ligand which has an affinity for non-covalent binding to a DHFR proteinaceous active site receptor moiety, a linking group, and the residue of an immunoreactive material together with a radioactive delivery agent comprised of the residue of a DHFR proteinaceous active site receptor moiety, a linking group, and a radioactive agent. These compositions comprise useful systems for the production of an amplification of delivery of the radioactive agent to tumor sites in the therapy and diagnostic imaging of cancer.

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This invention describes a non-radioactive targeting immunoreagent comprised of the residue of a proteinaceous active site of a dihydrofolate reductase enzyme (DHFR), a linking group, and the residue of an immunoreactive material together with a radioactive delivery agent comprised of the residue of a ligand which has an affinity for non-covalent binding to said DHFR receptor moiety, a linking group, and the residue of a radioactive agent. This invention also describes a non-radioactive targeting immunoreagent comprised of the residue of a ligand which has an affinity for non-covalent binding to a DHFR proteinaceous active site receptor moiety, a linking group, and the residue of an immunoreactive material together with a radioactive delivery agent comprised of the residue of a DHFR proteinaceous active site receptor moiety, a linking group, and a radioactive agent. These compositions comprise useful systems for the production of an amplification of delivery of the radioactive agent to tumor sites in the therapy and diagnostic imaging of cancer.

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IMMUNOREACTIVE REAGENTS EMPLOYING DIHYDROFOLATE REDUCTASE

Field of the Invention

This invention relates to the therapeutic treatment and diagnostic imaging of cancer by means of a tumor targeted sequential delivery system comprised of a primary non-radioactive targeting immunoreagent and a secondary radioactive delivery agent.

Background of the Invention

The various, currently available, radiolabeled immunoreactive proteins which are employed in diagnostic imaging and targeted therapeutic applications suffer from certain of the following disadvantages:

1) toxicity;

2) destruction or excretion of the reagent due to rapid catabolism or metabolism;

3) inadequate energy of radioactive emission which results in a low signal to noise ratio;

4) inefficient covalent bonding of the radioactive component with protein in conjugate preparation;

5) long plasma half-lives of currently available radionuclide-containing immunoreactive proteins result in prolonged exposure of normal tissue to damaging effects of radiation that can produce unacceptable toxic effects in otherwise normal and disease free tissues in the body, especially in those tissues and cells most sensitive to radiation damage, e.g., the stem cells of the bone marrow and gastrointestinal tract;

6) slow clearance of radionuclide from the body;

7) increasing the number of sites of incorporation of radionuclide or chelated radionuclide results in a

reduction in the capacity of the radionuclide-containing immunoreactive protein to bind to its target;

8) the number of chelating agents that can be attached to an immunoreactive protein is limited by the need to restrict chemical conjugations to sites removed from the immunoreactive recognition or binding sites of the protein;

9) the number of chelating agents that can be attached to an immunoreactive protein is limited by the number of available groups such as, for example, amino groups suitable for use in attachment of the chelating agents;

15) the number of chelating agents that can be attached to an immunoreactive protein is limited by the potential immunogenicity of the thus modified protein which, being highly derivatized, could be recognized by the immune system as haptenated;

16) the number of ionic radionuclides that can be associated with an immunoreactive protein is restricted by the number of sites of chelation available; and

17) radioimmunotherapy and diagnostic imaging with the various currently available radionuclide containing immunoreactive proteins can be less than optimal because these radiopharmaceuticals may bind to non-target normal tissue, which binding can result in undesirable toxicity to normal tissue during therapeutic applications as well as in high background signals during diagnostic imaging applications.

It is an object of the present invention to overcome the aforementioned disadvantages of the currently available radiolabeled immunoreactive proteins.

Summary of the Invention

The present invention is directed to systems which are useful in the therapeutic treatment and diagnostic imaging of tissue, particularly of cancerous tissue. For a disease such as cancer, such systems comprise a tumor targeted sequential delivery system comprised of a primary non-radioactive targeting immunoreagent and a secondary radioactive delivery agent.

In one embodiment, the present invention is directed to a non-radioactive targeting immunoreagent (sometimes hereinafter referred to as NRTIR) comprised of the residue of a receptor moiety, a linking group, and the residue of an immunoreactive material, which NRTIR is administered to a tissue of interest and will bind to sites on the surfaces of cells thereof.

In this embodiment, the present invention is also directed to a radioactive delivery agent (sometimes hereinafter referred to as RDA) comprised of the residue of a ligand which has an affinity for non-covalent binding to a receptor moiety, a linking group, and the residue of a radioactive agent. This RDA is administered to the environs of the tissue which contains said NRTIR bound thereto. In particular, the ligand residue of this RDA will non-covalently bind to the receptor of said NRTIR which is bound to the cells of said tissue of interest. Thus, an effective amount of radioactivity is provided to said tissue. RDA which is unbound to NRTIR can be removed rapidly from the environs of the tissue.

In one aspect of this embodiment (sometimes hereinafter referred to as System A), the present invention comprises an NRTIR comprised of the residue of a receptor moiety which is comprised of the residue of a proteinaceous active site of a dihydrofolate reductase

enzyme (sometimes hereinafter referred to as a DHFR), a linking group, and the residue of an immunoreactive material. In system A, the present invention also comprises an RDA comprised of a ligand which has an affinity for non-covalent binding to a DHFR receptor moiety, a linking group, and the residue of a radioactive agent.

Specifically, in System A, the present invention is directed to an NRTIR comprised of a residue of the proteinaceous active site of a dihydrofolate reductase enzyme, a linking group, and the residue of an immunoreactive material such as a tumor targeting antibody together with an RDA comprised of the residue of a ligand which has an affinity for non-covalent binding to said DHFR receptor moiety, a linking group, and the residue of a radioactive agent comprised of the residue of a chelating agent and a radionuclide.

The NRTIR of system A is comprised of (n) DHFR moieties, each of which can non-covalently bind an RDA comprised of the residue of a ligand with an affinity for non-covalent binding to a DHFR and of (m) radioactive agents where each of n and m is independently an integer greater than zero. The total number of radioactive agents capable of being bound per antigen is then the product of (n) multiplied by (m). This is in contrast to the binding to cell surface antigen of previously available radioimmunoconjugates comprised of an immunoreactive protein conjugated to (c) radioactive agents wherein the value of (c) is an integer greater than zero and is limited to the number of conjugations that can be performed on said immunoreactive protein while retaining the immunoreactivity for said antigen. This limit to the degree of modification of the immunoreactive protein also applies to the NRTIR of System A, and the value of (n) will be approximately the same as the value of (c).

Thus, in this aspect, the non-covalent binding of the RDA to the antigen-bound NRTIR of the present invention will amplify the maximum number of radioactive agents bound per antigen by a factor of approximately (m) over the maximum value (c) available in previously available radioimmunoconjugates.

In another embodiment, the present invention is directed to an NRTIR comprised of the residue of a ligand which exhibits an affinity for non-covalent binding to a receptor moiety, a linking group, and the residue of an immunoreactive material, which NRTIR is administered to a tissue of interest and will bind to sites on the surfaces of cells thereof.

In this embodiment, the present invention is also directed to an RDA comprised of the residue of a receptor moiety for which a ligand has an affinity for non-covalent binding, a linking group, and the residue of a radioactive agent, which RDA is administered to the environs of the tissue which contains the NRTIR of this embodiment bound thereto. In particular, the ligand of the RDA of this embodiment will non-covalently bind to the receptor of the NRTIR which is bound to the surface of the cells of said tissue of interest. Thus, an effective amount of radioactivity is provided to said tissue. RDA which is unbound to NRTIR can be removed rapidly from the environs of the tissue.

In particular, in one aspect of this other embodiment (sometimes hereinafter referred to as System B), the present invention comprises a NRTIR comprised of the residue of a ligand which has an affinity for non-covalent binding to a DHFR receptor moiety, a linking group, and the residue of an immunoreactive material. In system B the present invention also comprises an RDA comprised of the residue of a DHFR receptor moiety, a linking group, and the residue of a radioactive agent.

Specifically, in System B, the present invention is directed to a NRTIR comprised of the residue of a ligand which has an affinity for non-covalent binding to a DHFR receptor moiety, a linking group, and the residue of an immunoreactive material such as a tumor targeting antibody. In system B the present invention also comprises an RDA comprised of the residue of a DHFR receptor moiety, a linking group, and the residue of a radioactive agent comprised of the residue of a chelating agent and a radionuclide.

The NRTIR of System B is comprised of (n) residues of ligands that have an affinity for non-covalent binding to a DHFR, each of which can non-covalently bind an RDA comprised of a DHFR and of (m) radioactive agents where each of n and m is independently an integer greater than zero. The total number of radioactive agents capable of being bound per antigen is then the product of (n) multiplied by (m). This is in contrast to the binding to cell surface antigen of previously available radioimmunoconjugates comprised of an immunoreactive protein conjugated to (c) radioactive agents. In these conjugates the value of (c) is limited to the number of radioactive agents that can be linked or conjugated to the immunoreactive protein while retaining the immunoreactivity for said antigen. This limit to the degree of modification of the immunoreactive protein also applies to the NRTIR of System B, and the value of (n) will be approximately the same as the value of (c). Thus, in this aspect, the non-covalent binding of the RDA to the antigen-bound NRTIR of the present invention will amplify the maximum number of radioactive agents bound per antigen by a factor of approximately (m) over the maximum value (c) available in previously available radioimmunoconjugates.

The present invention is also directed to pharmaceutical and diagnostic compositions comprising an

NRTIR and a pharmaceutically acceptable carrier, and to pharmaceutical and diagnostic compositions comprising an RDA and a pharmaceutically acceptable carrier.

The present invention is further directed to therapeutic methods comprising the administration, in vitro or in vivo, of a therapeutically effective amount of NRTIR to the environs of a tissue of interest of a patient undergoing such therapy, followed after the lapse of an effective period of time by the subsequent administration of a therapeutically effective amount of RDA to said tissue. During the time between administrations of NRTIR and RDA said NRTIR binds to sites on cells of the target tissue and unbound NRTIR is removed from the environs of said tissue.

The present invention is further directed to diagnostic imaging methods comprising the sequential administration, in vitro or in vivo, of a diagnostic imaging effective amount of an NRTIR to the environs of a tissue of interest of a patient undergoing such diagnostic imaging, followed after a lapse of an effective period of time by the subsequent administration of a diagnostic imaging effective amount of RDA to said tissue. During said effective period of time said NRTIR will bind to sites on cells of said tissue of interest and unbound NRTIR will be removed from the environs of the tissue. Subsequently, after an effective time, an image of all or part of said tissue of interest is obtained.

The present invention provides advantages compared to currently available targeting immune reagents. For example:

the total amount of a therapeutically effective amount and of a diagnostic imaging effective amount of radioactive agent delivered to a tissue site can be achieved with specificity and in amplification over that

which can be otherwise achieved with currently available targeting immune reagents;

sequential delivery to target tissue of the NRTIR and the RDA of this invention can reduce the exposure of non-targeted tissues to damage from radiation;

the non-covalent binding of the ligand to the receptor occurs with high affinity and is selective;

the NRTIR and the RDA can be used in both therapeutic and diagnostic imaging applications;

the above-described NRTIR can accumulate at a tumor tissue site in vivo while it is not substantially accumulated at normal tissue sites;

the in vivo residence half life of the above-described NRTIR is long enough to permit its accumulation at a tumor site;

the in vivo residence half life of the above-described RDA is shorter than the residence half life of the above-described NRTIR;

RDA that does not bind to cell associated NRTIR is rapidly cleared from the patient;

with respect to the same degree of modification of a targeting immunoreagent directly conjugated by a radionuclide or by a chelate containing a radionuclide in currently available radioimmunoconjugates, an amplification of the number of radionuclides per site of modification per targeting immune reagent can be obtained using the materials and methods of this invention;

the NRTIR can comprise a wide variety of immunoreactive groups, linking groups, and DHFR active site residues in System A, and a wide variety of immunoreactive groups, linking groups, and ligand residues which have an affinity for non-covalent binding to DHFR active site residues in System B;

the RDA can comprise a wide variety of spacing, linking and chelating groups, radionuclides, and ligand

residues which ligands have an affinity for non-covalent binding to DHFR in System A, and a wide variety of spacing, linking and chelating groups, radionuclides, and DHFR active site residues in System B; and

a wide variety of compositions of matter with a wide variety of sizes and molecular weights and having a specificity for accumulation in tumors can be prepared in accordance with this invention.

Other advantageous features of this invention will become readily apparent upon reference to the following description of the preferred embodiments.

Description of Preferred Embodiments

In preferred embodiments, the above-described non-radioactive targeting immunoreagent (NRTIR) and radioactive delivery agent (RDA) are comprised of moieties represented in System A (4 systems) and System B (4 systems) below:

SYSTEM A

	Non-Radioactive Targeting ImmunoReagent NRTIR	Radioactive Delivery Agent RDA
1	Immunoreactive group + (linking group + receptor)$_n$	Ligand + (chelating agent + radionuclide)$_m$
2	Z-(L_1-Rec)$_n$	D-(L_2-Q-M)$_m$
3	Z-(L_1-Rec)$_n$	Trimethoprim-(L_2-Q-M)$_m$
4	Z-(L_1-Rec)$_n$	Methotrexate-(L_2-Q-M)$_m$

SYSTEM B

	Non-Radioactive Targeting ImmunoReagent NRTIR	Radioactive Delivery Agent RDA
1	Immunoreactive group + (linking group + ligand)$_n$	Receptor + (linking group + chelating agent + radionuclide)$_m$
2	Z-(L_1-DHFR ligand)$_n$	Rec-(L_2-Q-M)$_m$
3	Z-(L_1-TMP)$_n$	Rec-(L_2-Q-M)$_m$
4	Z-(L_1-MTX)$_n$	Rec-(L_2-Q-M)$_m$

wherein:

Z is the residue of an immunoreactive group;

Rec is the residue of a receptor, preferably a DHFR;

D is the residue of a ligand that has an affinity for non-covalent binding to a receptor, preferably to a DHFR receptor;

DHFR ligand is the residue of a ligand that has an affinity for non-covalent binding to a DHFR active site;

TMP is the residue of a ligand comprised of a trimethoprim analog;

MTX is the residue of a ligand comprised of a methotrexate analog;

L_1 and L_2 are each independently the residue of a linking group that may independently contain a spacing group;

Q is the residue of a chelating group;

M is a radionuclide; and

n and m are each independently an integer greater than zero.

Preferred embodiments of these materials are further described below.

The term "residue" is used herein in context with a chemical entity. Said chemical entity is comprised of, for example, a ligand, or a trimethoprim analog, or a methotrexate analog, or a receptor moiety, or a proteinaceous active site of a dihydrofolate reductase enzyme, or a DHFR, or a chelating group, or a radioactive agent, or a linking group, or a protein reactive group, or an immunoreactive group, or an immunoreactive material, or an immunoreactive protein, or an antibody, or an antibody fragment, or a cross-linking agent such as a heterobifunctional cross-linking agent, or a spacing group. The term "residue" is defined as that portion of said chemical entity which exclusively remains when one or more chemical bonds of which said chemical entity is otherwise comprised when considered as an independent chemical entity, is altered, modified, or replaced to comprise one or more covalent bonds to one or more other chemical entities. Thus, for example, in one aspect in System A and in System B, the residue of a chelating group is comprised of a chelating group which is at least monovalently modified through attachment to the residue of another chemical entity such as, for example, to the residue of a linking group.

In both System A and System B the immunoreactive group, Z, can be selected from a wide variety of naturally occurring or synthetically prepared materials, including, but not limited to enzymes, amino acids, peptides, polypeptides, proteins, lipoproteins, glycoproteins, lipids, phospholipids, hormones, growth factors, steroids, vitamins, polysaccarides, viruses, protozoa, fungi, parasites, rickettsia, molds, and components thereof, blood components, tissue and organ components, pharmaceuticals, haptens, lectins, toxins,

nucleic acids (including oligonucleotides), antibodies (monoclonal and polyclonal), anti-antibodies, antibody fragments, antigenic materials (including proteins and carbohydrates), avidin and derivatives thereof, biotin and derivatives thereof, and others known to one skilled in the art. In addition, an immunoreactive group can be any substance which when presented to an immunocompetent host will result in the production of a specific antibody capable of binding with that substance, or the antibody so produced, which participates in an antigen-antibody reaction.

Preferred immunoreactive groups are antibodies and various immunoreactive fragments thereof, as long as they contain at least one reactive site for reaction with the reactive groups on the residue of the receptor moiety in System A or ligand species in System B or with linking groups (L) as described herein. That site can be inherent to the immunoreactive species or it can be introduced through appropriate chemical modification of the immunoreactive species. In addition to antibodies produced by the techniques outlined above, other antibodies and proteins produced by the techniques of molecular biology are specifically included.

As used herein, the term "antibody fragment" refers to an immunoreactive material which comprises a residue of an antibody, which antibody characteristically exhibits an affinity for binding to an antigen. The term affinity, as used herein, refers to the thermodynamic expression of the strength of interaction or binding between an antibody combining site (or other ligand) and an antigenic determinant (or receptor)and, thus, of the sterochemical compatibility between them; as such it is the expression of the equilibrium or association constant for the antibody-antigen (or ligand-receptor) interaction. Antibody fragments exhibit at least a percentage of said affinity for binding to said antigen,

said percentage being in the range of 0.001 per cent to 1,000 per cent, preferably 0.01 per cent to 1,000 per cent, more preferably 0.1 per cent to 1,000 per cent, and most preferably 1.0 per cent to 1,000 per cent, of the relative affinity of said antibody for binding to said antigen.

An antibody fragment can be produced from an antibody by a chemical reaction comprising one or more chemical bond cleaving reactions; by a chemical reaction comprising one or more chemical bond forming reactions employing as reactants one or more chemical components selected from a group comprised of amino acids, peptides, carbohydrates, linking groups as defined herein, spacing groups as defined herein, protein reactive groups as defined herein, and antibody fragments such as are produced as described herein; and by a molecular biological process, a bacterial process, or by a process comprised of a genetic engineering of antibody genes.

An antibody fragment can be derived from an antibody by a chemical reaction comprised of one or more of the following reactions:

(a) cleavage of one or more chemical bonds of which an antibody is comprised, said bonds being selected from, for example, carbon-nitrogen bonds, sulfur-sulfur bonds, carbon-carbon bonds, carbon-sulfur bonds, and carbon-oxygen bonds, and wherein the method of said cleavage is selected from:

(i) a catalysed chemical reaction comprising the action of a biochemical catalyst such as an enzyme such as papain or pepsin which to those skilled in the art is known to produce antibody fragments commonly referred to as Fab and Fab'2, respectively;

(ii) a catalysed chemical reaction comprising the action of an electrophilic chemical catalyst such as a

hydronium ion which, for example, favorably occurs at a pH equal to or greater than 7;

(iii) a catalysed chemical reaction comprising the action of a nucleophilic catalyst such as a hydroxide ion which, for example, favorably occurs at a pH equal to or greater than 7; and

(iv) a chemical reaction comprised of a substitution reaction employing a reagent which is consumed in a stoichiometric manner such as a substitution reaction at a sulfur atom of a disulfide bond by a reagent comprised of a sulfhydryl group;

(v) a chemical reaction comprised of a reduction reaction such as the reduction of a disulfide bond; and

(vi) a chemical reaction comprised of an oxidation reaction such as the oxidation of a carbon-oxygen bond of a hydroxyl group or the oxidation of a carbon-carbon bond of a vicinal diol group such as occurs in a carbohydrate moiety; or

(b) formation of one or more chemical bonds between one or more reactants, such as formation of one or more covalent bonds selected from, for example, carbon-nitrogen bonds (such as, for example, amide bonds, amine bonds, hydrazone bonds, and thiourea bonds), sulfur-sulfur bonds such as disulfide bonds, carbon-carbon bonds, carbon-sulfur bonds, and carbon-oxygen bonds, and employing as reactants in said chemical bond formation one or more reagents comprised of amino acids, peptides, carbohydrates, linking groups as defined herein, spacing groups as defined herein, protein reactive groups as defined herein, and antibody fragments such as are produced as described in (a), above; or

(c) an antibody fragment can be derived by formation of one or more non-covalent bonds between one or more reactants. Such non-covalent bonds are comprised of hydrophobic interactions such as occur in an aqueous medium between chemical species that are

independently comprised of mutually accessible regions of low polarity such as regions comprised of aliphatic and carbocyclic groups, and of hydrogen bond interactions such as occur in the binding of an oligonucletide with a complementary oligonucletide; or

(d) an antibody fragment can be produced as a result of the methods of molecular biology or by genetic engineering of antibody genes, for example, in the genetic engineering of a single chain immunoreactive group or a Fv fragment.

An antibody fragment can be produced as a result a combination of one or more of the above methods.

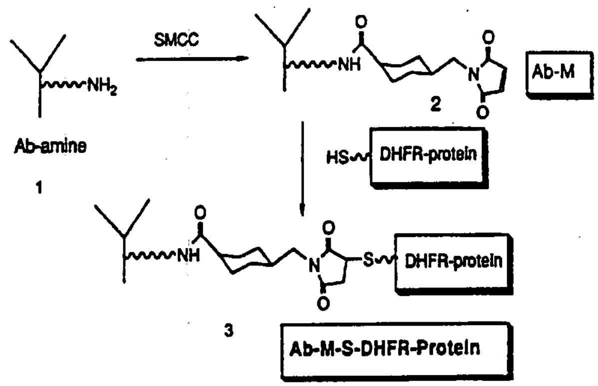
In certain embodiments, the immunoreactive group can be an enzyme which has a reactive group for attachment to the receptor moiety in System A or ligand species in System B or to a linking group as described below. Representative enzymes include, but are not limited to, aspartane, aminotransaminase, alanine aminotransaminase, lactate dehydrogenase, creatine phosphokinase, gamma glutamyl transferase, alkaline acid phosphatase, prostatic acid phosphatase, horseradish peroxidase and various esterases.

If desired, the immunoreactive group can be modified or chemically altered to provide reactive groups for attaching to the residues of the receptor moiety in System A or ligand species in System B or to a linking group as described below by techniques known to those skilled in the art. Such techniques include the use of linking moieties and chemical modification such as described in WO-A-89/02931 and WO-A-89/2932, which are directed to modification of oligonucleotides, and U.S. Patent No. 4,719,182.

Two highly preferred uses for the compositions of this invention are for the diagnostic imaging of tumors and the radiological treatment of tumors. Preferred immunological groups therefore include antibodies

(sometimes hereinafter referred to as Ab) to tumor-associated antigens. Specific non-limiting examples include B72.3 and related antibodies (described in U.S. Patent Nos. 4,522,918 and 4,612,282) which recognize colorectal tumors, 9.2.27 and related anti-melanoma antibodies, D612 and related antibodies which recognize colorectal tumors, UJ13A and related antibodies which recognize small cell lung carcinomas, NRLU-10 and related antibodies which recognize small cell lung carcinomas and colorectal tumors (Pan-carcinoma), 7E11C5 and related antibodies which recognize prostate tumors, CC49 and related antibodies which recognize colorectal tumors, TNT and related antibodies which recognize necrotic tissue, PR1A3 and related antibodies which recognize colon carcinoma, ING-1 and related antibodies, which are described in International Patent Publication WO-A-90/02569, B174 and related antibodies which recognize squamous cell carcinomas, B43 and related antibodies which are reactive with certain lymphomas and leukemias, and anti-HLB and related monoclonal antibodies. An especially preferred antibody is ING-1.

Scheme 1



As used herein, the term "receptor" refers to a chemical group in a molecule which comprises an active site in said molecule, or to an array of chemical groups in a molecule which comprise one or more active sites in said molecule, or to a molecule comprised of one or more chemical groups or one or more arrays of chemical groups, which group or groups or array of groups comprise one or more active sites in said molecule. An "active site" of a receptor has a specific capacity to bind to or has an affinity for binding to a ligand. With respect to use with the term "receptor" or with the term "active site in a receptor", the term "ligand" as used herein refers to a molecule comprised of a specific chemical group or a specific array of chemical groups which molecule, group, or array of groups is complementary to and has a specific affinity for binding to a receptor, especially to an active site in a receptor. Examples of receptors include enzymes which catalyze chemical reactions, and cell surface receptors which bind hormones and drugs. The sites of specific

binding of substrates for said enzymes and of hormones and drugs of said cell surface receptors are examples of active sites of said receptors, and the respective substrates, hormones, and drugs are examples of ligands for said receptors.

Preferred receptors (Rec) in System A and System B are comprised of the residue of an active site of an enzyme, and preferred ligands in System A and System B are comprised of the residue of a substrate for said active site of said enzyme.

An especially preferred receptor is comprised of the active site of any protein having dihydrofolate reductase (DHFR) activity. Said DHFR can be isolated, in whole or in part, from any source and used in this invention without further modification, or it can be chemically modified before or after isolation for use in this invention, or it can be modified by well known techniques of molecular biology and isolated for use in this invention, or said molecular biology modified DHFR can be chemically modified before or after isolation for use in this invention as long as the DHFR active site is maintained in such use.

According to the 1992 Sigma Chemical Company catalog on page 350, DHFR activity is defined in terms of units; one said unit of DHFR enzyme activity is defined as the amount of material needed to convert 1.0 micromole of 7,8-dihydrofolate and NADPH to 5,6,7,8-tetrahydrofolate and NADP per minute at pH 6.5 and 25°C. Thus, as used herein, a chemical species comprised of a group or array of groups that catalyze the conversion of 7,8-dihydrofolate and NADPH to 5,6,7,8-tetrahydrofolate and NADP has DHFR activity and such group or array of groups comprises a dihydrofolate reductase active site, i.e., a DHFR. In addition, the modification of a DHFR by known techniques of molecular biology or by chemical modification may reduce the protein's ability to

catalyze the conversion of 7,8-dihydrofolate and NADPH to 5,6,7,8-tetrahydrofolate and NADP yet retain the protein's capacity to bind a ligand.

Examples of ligands that have an affinity for binding to the active site in said DHFR include 7,8-dihydrofolate, derivatives and analogs of 7,8-dihydrofolate, and residues of said derivatives and analogs which may be agonists or antagonists of DHFR activity with respect to 7,8-dihydrofolate. Preferred examples of ligands which exhibit an affinity for binding to a DHFR are comprised of antifolate drugs as described in "Principals of Drug Action, The Basis of Pharmacology", Third Edition, Churchill Livingstone, New York, 1990, Pratt W. B. and Taylor P., Editors, page 623 which are antagonists of DHFR activity with respect to 7,8-dihydrofolate, and of inhibitors of DHFR activity as described in Blaney, J. M., Hansch, C., Silipo, C., and Vittoria, A., Chemical Reviews, (1984), Vol. 84, pp 333-407. Additional preferred examples are comprised of derivatives and analogs of said antifolate drugs and inhibitors, and residues of said derivatives and said analogs of said antifolate drugs and inhibitors. Especially preferred ligands which exhibit an affinity for binding to a DHFR are comprised of residues of the antifolate drugs, pyrimethamine, methotrexate, tetroxoprim, and trimethoprim. Most preferred are comprised of residues of the antifolate drugs, methotrexate and trimethoprim.

In one aspect in System A and in System B, the DHFR is comprised of a non-human protein, preferably comprised of a bacterial or protozoal protein, and more preferably of a bacterial protein. Preferably, said DHFR is comprised of protein derived from S. aureus or from E. Coli. More preferably, said DHFR is comprised of protein derived from E. Coli (strain CV634) infected

with the plasmid pCV29 which harbors the E. coli DHFR gene.

In another aspect in System A and in System B, the DHFR is comprised of a human protein. Preferably, said DHFR is comprised of a recombinant human protein. More preferably, said DHFR is comprised of a recombinant human protein which is modified by genetic engineering techniques, which modifications comprise the independent incorporation, substitution, insertion, and deletion of specific amino acids in a peptide sequence of said protein. Yet more preferably, the DHFR comprised of a thus modified recombinant human protein is comprised of an active site which has an affinity for binding to substrates, which affinity is greater than the affinity of natural human protein for said substrates. Even more preferably, the DHFR comprised of a thus modified recombinant human protein is comprised of an active site which has an affinity for binding to residues of the antifolate drugs, methotrexate and trimethoprim, which affinity is greater than the affinity of natural human protein for binding to the residues of said antifolate drugs. Most preferrably, the DHFR comprised of a thus modified recombinant human protein is comprised of an active site which has an affinity for binding to residues of the antifolate drug, trimethoprim, which affinity is greater than the affinity of natural human protein for binding to the residues of said antifolate drug.

In yet another aspect, the Z-L-X of System A is comprised of a fusion protein. As used herein, the term "fusion protein" refers to a genetically engineered material comprised of a protein whose coding region is comprised of the coding region of a residue of a first protein fused, in frame, to the coding region of a residue of a second protein. Preferably, said fusion protein is comprised of a protein whose coding region is

comprised of the coding region of a residue of an immunoreactive reagent fused, in frame, to the coding region of one or more residues of a DHFR. Thus, preferably, said fusion protein is comprised of a residue of an immunoreactive reagent fused to one or more residues of a DHFR. In a prefered embodiment, said fusion protein is comprised of residues of DHFR fused to an immunoglobulin heavy chain in the CH_1 region, such that when combined with an appropriate light chain the said fusion protein comprises an Fab fragment linked to one or more DHFR. In another preferred embodiment, said fusion protein can be comprised of one or more DHFR fused to an immunoglobulin heavy chain in the CH_2 or in the CH_3 region. In yet another preferred embodiment, said fusion protein, when comprised of an immunoglobulin light chain, can be comprised of a Fab'2 fragment linked to one or more DHFR. In still another preferred embodiment, said fusion protein can be comprised of one or more DHFR fused to the C-terminal end of an immunoglobulin single-chain construct and thus be comprised of an Fv fragment linked to one or more DHFR.

The above genetically engineered fusion protein comprising Z-L-X of System A can be comprised a protein whose coding region is independently comprised of the coding region of a residue of a human or of a non-human first protein fused, in frame, to the coding region of a residue of a human or non-human second protein. Preferably, said coding regions are independently human and bacterial or modified by genetic engineering techniques as above. More preferably, the fusion protein is comprised of a protein whose coding region is comprised of the coding region of a residue of a human immunoreactive reagent fused, in frame, to the coding region of one or more residues of a bacterial or human DHFR or a genetically engineered modified bacterial or human DHFR. Yet more preferably, the fusion protein is

comprised of a thus modified recombinant human DHFR comprised of an active site which has an affinity for binding to a ligand, which affinity is greater than the affinity of natural human protein for said ligand.

Even more preferably, the fusion protein is comprised of a thus modified recombinant human DHFR comprised of an active site which has an affinity for binding to a ligand comprised of a residue of an antifolate drug such as methotrexate or trimethoprim, which affinity is greater than the affinity of natural human protein for binding to the residue of said ligand. Most preferably, the fusion protein is comprised of a thus modified recombinant human DHFR comprised of an active site which has an affinity for binding to a ligand comprised of a residue of trimethoprim, which affinity is greater than the affinity of natural human protein for binding to the residue of said ligand.

The binding of a ligand to a receptor can comprise the formation of a covalent bond or it can comprise a non-covalent interaction. Preferably, in this invention the binding of a ligand to a receptor comprises a non-covalent interaction, sometimes herein referred to as non-covalent binding.

In System A, the DHFR is covalently linked, i.e., conjugated, to an immunoreactive group, preferably to an antibody or to an antibody fragment, most preferably to ING-1, to form the NRTIR [i.e., $Z-(L_1-Rec)_n$] of the System.

In System B, in one embodiment, the DHFR as a component of a radioactive delivery agent [i.e., an RDA, $Rec-(L_2-Q-M)_m$] is attached to one or more chelating groups, each by means of a linking group, and the chelating group is associated with a radionuclide. Preferably the chelating group is TMT (described hereinbelow), the linking group is as described below, and the radionuclide is ^{90}Y.

In another embodiment, the RDA in System B is comprised of a DHFR that contains one or more radionuclides that are covalently attached, either directly to one or more components of the DHFR or to one or more components that are attached by a linking group as described below to the DHFR. Preferably, said covalently attached radionuclide is a radioisotope of iodine attached to an aromatic ring-containing moiety.

In another embodiment, the RDA in System B is comprised of a DHFR that contains one or more radionuclides that are covalently attached, either directly to one or more components of the DHFR or to one or more components that are attached by a linking group as described below to the DHFR. Preferably, said covalently attached radionuclide is selected from a radioisotope of technicium and rhenium attached to a group comprised of a sulfur atom.

In System A, chemical conjugation can be achieved, for example, by a technique comprising the use of a linking group (L_1) which is introduced through modification of, for example, a site on an immunoreactive group. The introduction of activated groups such as activated ethylene groups (e.g., maleimide groups) on to amine groups such as lysine epsilon-amines of a protein is represented in Scheme 1. Other techniques include the use of heterobifunctional linking moieties and chemical modifications such as the examples described in U. S. Patent No. 4,719,182. Additionally, those chemicals such as SMCC which are commonly commercially available, for example, from Pierce Chemical Company are included as non-limiting examples.

In both System A and System B in one aspect, chemical conjugation is otherwise achieved by using a linking group (L_1 and L_2, respectively) which is introduced through mild reduction of the DHFR (or of the

DHFR modified by reagents which contain disulfide bonds) with a reducing reagent such as dithiothreitol to produce sulfhydryl (SH) sites in the reduced DHFR protein moiety. In System A, addition of the thus reduced DHFR protein moiety to the above described maleimide modified antibody (Ab-M) results in an antibody/receptor conjugate (Ab-M-S-DHFR protein) linked together by one or more thioether bonds. Additionally, those chemicals which are commonly commercially available, for example, from Pierce Chemical Company and the like which are useful in the covalent attachment of two proteins are included as non-limiting examples in the coupling of DHFR to antibody in System A.

In System B, addition of the thus reduced DHFR protein moiety to a chelating agent which contains a precursor of a linking group comprised of an activated ethylene group such as a maleimide group results in a DHFR/chelating agent conjugate linked together by one or more thioether bonds. Similarly, addition of the thus reduced immunoreactive protein moiety to the residue of a ligand which contains a precursor of a linking group comprised of an activated ethylene group such as a maleimide group results in a immunoreactive protein moiety/ligand conjugate linked together by one or more thioether bonds.

In System A, other groups are useful in the coupling of the immunoreactive material to the receptor moiety, particularly if the above reagents are utilized. Suitable reactive sites on the immunoreactive material and on the receptor moiety include:

amine sites of lysine;
terminal peptide amines;
carboxylic acid sites, such as are available in aspartic acid and glutamic acid;
sulfhydryl sites;
carbohydrate sites;

activated carbon-hydrogen and carbon-carbon bonds which can react through insertion via free radical reaction or nitrene or carbene reaction of a so activated residue;
sites of oxidation;
sites of reduction;
aromatic sites such as tyrosine; and
hydroxyl sites.

In System A, the ratio of DHFR to immunoreactive group such as an antibody can vary widely from about 0.5 to 10 or more. In bulk, mixtures comprised of immunoreactive groups which are unmodified and immunoreactive groups which are modified with DHFR are also suitable. Such mixtures can have a bulk ratio of DHFR to immunoreactive group of from about 0.1 to about 10.

In System A, in preferred embodiments, the mole ratio of DHFR to immunoreacative group is from about 1:1 to about 6:1. It is specifically contemplated that with knowledge of the DNA sequence that encodes DHFR, especially bacterial and human DHFR, a fusion protein can be made between the antibody and the DHFR, or portions thereof, through the use of genetic engineering techniques. It is specifically contemplated that in all of these compositions of DHFR bound to antibody, DHFR retains a capacity to bind to the ligands described in the invention.

In System B, the ratio of ligand to immunoreactive group such as an antibody can vary widely from about 0.5 to 10 or more. In bulk, mixtures comprised of immunoreactive groups which are unmodified and immunoreactive groups which are modified with ligand are also suitable. Such mixtures can have a bulk ratio of ligand to immunoreactive group of from about 0.1 to about 10. In preferred embodiments, the mole ratio of ligand to immunoreactive group is from about 1:1 to about 6:1.

In System A, following the linking of the immunoreactive group, preferably of an antibody or an antibody fragment, to DHFR, the conjugate is purified by passage of the material through a gel permeation column such as Superose 6 using an appropriate elution buffer or by elution from a HPLC column such as a Shodex WS-803F size exclusion column. Both these methods separate the applied materials by molecular size resulting in the elution of the antibody/DHFR conjugate in a different fraction from any residual non-conjugated DHFR.

In System A, the concentrations of the antibody in the conjugate solutions are determined by the Bradford (BioRad Catalog # 500-0001) method using bovine immunoglobulin as the protein standard.

In System B, following the linking of the immunoreactive group, preferably of an antibody or an antibody fragment, to the residue of a ligand, the conjugate is purified by passage of the material through a gel permeation column such as Superose 6 using an appropriate elution buffer or by elution from a HPLC column such as a Shodex WS-803F size exclusion column. Both these methods separate the applied materials by molecular size resulting in the elution of the antibody/ligand conjugate in a different fraction from any residual non-conjugated ligand.

In System B, the concentrations of the antibody in the conjugate solutions are determined by the BioRad protein assay using bovine immunoglobulin as the protein standard.

In System A, the ability of the antibody to bind to its target antigen following conjugation to DHFR can be assayed by ELISA or flow cytometry. A 30 cm x 7.5 mm TSK-G3000SW size-exclusion HPLC column (Supelco) fitted with a guard column of the same material can be used to determine the amount of aggregation in the final conjugate.

In System B, the ability of the antibody to bind to its target antigen following conjugation to the residue of a ligand can be assayed by ELISA or flow cytometry. A 30 cm x 7.5 mm TSK-G3000SW size-exclusion HPLC column (Supelco) fitted with a guard column of the same material can be used to determine the amount of aggregation in the final conjugate.

In System A, the dihydrofolate reductase enzymic activity of the antibody-associated DHFR can be assayed by following the oxidation of pyridine nucleotides during the reduction of folate to tetrahydrofolate by DHFR as described by Mathews and Huennekens (J. Biol. Chem. 238, 3436-3442 (1963)). This method can also be used to assay the DHFR inhibitory effects of the novel DHFR binding ligands which are modified to include chelating agents as described in this invention.

In System B, the dihydrofolate reductase enzymic activity of the chelating agent-associated DHFR can be assayed by following the oxidation of pyridine nucleotides during the reduction of folate to tetrahydrofolate by DHFR as described by Mathews and Huennekens (J. Biol. Chem. 238, 3436-3442 (1963)). This method can also be used to assay the DHFR inhibitory effects of the novel ligands which have an affinity for binding to DHFR and which are linked to immunoreactive groups as described in this invention.

L_1 and L_2 in System A and System B are independently a chemical bond or the residue of a linking group. In one aspect, the phrase "residue of a linking group" as used herein refers to a moiety that remains, results, or is derived from the reaction of a protein reactive group with a reactive site on a protein. The phrase "protein reactive group" as used herein refers to any group which can react with functional groups typically found on proteins. However, it is specifically contemplated that such protein

reactive groups can also react with functional groups typically found on relevant nonprotein molecules. Thus, in one aspect the linking groups L_1 and L_2 useful in the practice of this invention derive from those groups which can react with any relevant molecule "Z" or "Rec" as described above containing a reactive group, whether or not such relevant molecule is a protein, to form a linking group. In one aspect, preferred linking groups thus formed include the linking group, L_1, between the immunoreactive group, "Z", and the DHFR active site containing species, "Rec", in the NRTIR System A; the linking group, L_1, between the immunoreactive group, "Z", and DHFR ligand species (e.g., "TMP" or "MTX") in the NRTIR in System B; and the linking group, L_2, between the DHFR active site containing species, "Rec", and the chelating agent, "Q", in the RDA in System B; and between the DHFR ligand species (e.g., "TMP" or "MTX") and the chelating agent, "Q", in the RDA in System A.

Preferred linking groups are derived from protein reactive groups selected from but not limited to:

(1) a group that will react directly with amine, alcohol, or sulfhydryl groups on the immunoreactive protein or biological molecule containing the reactive group, for example, active halogen containing groups including, for example, chloromethylphenyl groups and chloroacetyl [$ClCH_2C(=O)-$] groups, activated 2-(leaving group substituted)-ethylsulfonyl and ethylcarbonyl groups such as 2-chloroethylsulfonyl and 2-chloroethylcarbonyl; vinylsulfonyl; vinylcarbonyl; epoxy; isocyanato; isothiocyanato; aldehyde; aziridino; succinimidoxycarbonyl; activated acyl groups such as carboxylic acid halides; mixed anhydrides and the like; and other groups known to be useful in conventional photographic gelatin hardening agents;

(2) a group that can react readily with modified proteins or biological molecules containing the immunoreactive group, i.e., proteins or biological molecules containing the immunoreactive group modified to contain reactive groups such as those mentioned in (1) above, for example, by oxidation of the protein to an aldehyde or a carboxylic acid, in which case the "linking group" can be derived from protein reactive groups selected from amino, alkylamino, arylamino, hydrazino, alkylhydrazino, arylhydrazino, carbazido, semicarbazido, thiocarbazido, thiosemicarbazido, sulfhydryl, sulfhydrylalkyl, sulfhydrylaryl, hydroxy, carboxy, carboxyalkyl and carboxyaryl. The alkyl portions of said linking groups can contain from 1 to about 20 carbon atoms. The aryl portions of said linking groups can contain from about 6 to about 20 carbon atoms; and

(3) a group that can be linked to the protein or biological molecule containing the immunoreactive group, or to the modified protein as noted in (1) and (2) above by use of a crosslinking agent. The residues of certain useful crosslinking agents, such as, for example, homobifunctional and heterobifunctional gelatin hardeners, bisepoxides, and bisisocyanates can become a part of, i.e., a linking group in, for example, the protein-(DHFR active site-containing species) conjugate in System A during the crosslinking reaction. Other useful crosslinking agents, however, can facilitate the crosslinking, for example, as consumable catalysts, and are not present in the final conjugate. Examples of such crosslinking agents are carbodiimide and carbamoylonium crosslinking agents as disclosed in U.S. Patent No. 4,421,847 and the ethers of U.S. Patent No. 4,877,724. With these crosslinking agents, one of the reactants such as the immunoreactive group must have a carboxyl group and the other such as the oligonucleotide

containing species must have a reactive amine, alcohol, or sulfhydryl group. In amide bond formation, the crosslinking agent first reacts selectively with the carboxyl group, then is split out during reaction of the thus "activated" carboxyl group with an amine to form an amide linkage between, for example, the protein and DHFR active site containing species, thus covalently bonding the two moieties. An advantage of this approach is that crosslinking of like molecules, e.g., proteins with proteins or DHFR active site containing species with themselves is avoided, whereas the reaction of, for example, homo-bifunctional crosslinking agents is nonselective and unwanted crosslinked molecules are obtained.

Preferred useful linking groups are derived from various heterobifunctional cross-linking reagents such as those listed in the Pierce Chemical Company Immunotechnology Catalog - Protein Modification Section, (1991 and 1992). Useful non-limiting examples of such reagents include:

Sulfo-SMCC	Sulfosuccinimidyl 4-(N-maleimidomethyl)cyclohexane-1-carboxylate.
Sulfo-SIAB	Sulfosuccinimidyl (4-iodoacetyl)aminobenzoate.
Sulfo-SMPB	Sulfosuccinimidyl 4-(p-maleimidophenyl)butyrate.
2-IT	2-Iminothiolane.
SATA	N-Succinimidyl S-acetylthioacetate.

In addition to the foregoing description, the linking groups, in whole or in part, can also be comprised of and derived from complementary sequences of nucleotides and residues of nucleotides, both naturally occurring and modified, preferably non-self-associating oligonucleotide sequences. Particularly useful, non-limiting reagents for incorporation of modified nucleotide moieties containing reactive functional groups, such as amine and sulfhydryl groups, into an oligonucleotide sequence are commercially available from, for example, Clontech Laboratories Inc. (Palo Alto California) and include Uni-Link AminoModifier (Catalog # 5190), Biotin-ON phosphoramidite (Catalog # 5191), N-MNT-C6-AminoModifier (Catalog # 5202), AminoModifier II (Catalog # 5203), DMT-C6-3'Amine-ON (Catalog # 5222), C6-ThiolModifier (Catalog # 5211), and the like. In one aspect, linking groups of this invention are derived from the reaction of a reactive functional group such as an amine or sulfhydryl group as are available in the above Clontech reagents, one or more of which has been incorporated into an oligonucleotide sequence, with, for example, one or more of the previously described protein reactive groups such as heterobifunctional protein reactive groups, one or more of which has been incorporated into, for example, an immune reactive agent or DHFR active site containing moiety of this invention.

In the NRTIR of System A, complementary oligonucleotide sequences are attached to two components of the conjugate, respectively, one sequence to the immune reactive agent and the complementary oligonucleotide sequence to the DHFR active site containing moiety. The hybrid formed between the two complementary oligonucleotide sequences then comprises the linking group between the immune reactive agent and the DHFR active site containing moiety.

In the RDA of System B, the complementary oligonucleotide sequences are attached to two components of the conjugate, one sequence to the residue comprised of one or more chelating agents and the complementary oligonucleotide sequence to the DHFR active site containing moiety. The hybrid formed between the two complementary oligonucleotide sequences then comprises the linking group between the DHFR active site containing moiety and the component comprised of one or more chelating agents.

In System B, of course, two or more copies of the same oligonucleotide sequence can be linked, for example, in tandem to one DHFR active site containing moiety and a complementary oligonucleotide sequence comprised of multiple chelating agents can be added. The multiple hybrids formed between the two complementary oligonucleotide sequences then comprises the linking group between the DHFR active site containing moiety and multiple chelating agents.

Likewise, in System B, the residue of one or more ligands which have an affinity for non-covalent binding to DHFR can be attached to the immunoreactive group using complementary oligonucleotide hybrids as described above.

In System A, analogously, multiple DHFR sequences can be attached to the immunoreactive protein. Likewise, in System A, one or more ligands which have an affinity for non-covalent binding to DHFR can be attached to multiple chelating agents using complementary oligonucleotide hybrids as described above.

Q in System A and in System B represents the residue of a chelating group. The chelating group of this invention can comprise the residue of one or more of a wide variety of chelating agents that can have a radionuclide associated therewith. As is well known, a

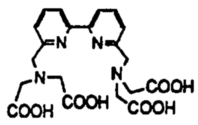
chelating agent is a compound containing donor atoms that can combine by coordinate bonding with a metal atom to form a cyclic structure called a chelation complex or chelate. This class of compounds is described in the Kirk-Othmer Encyclopedia of Chemical Technology, Vol. 5, 339-368.

The residues of suitable chelating agents can be independently selected from polyphosphates, such as sodium tripolyphosphate and hexametaphosphoric acid; aminocarboxylic acids, such as ethylenediaminetetraacetic acid, N-(2-hydroxyethyl)ethylene-diaminetriacetic acid, nitrilotriacetic acid, N,N-di(2-hydroxyethyl)glycine, ethylenebis(hydroxyphenylglycine) and diethylenetriamine pentacetic acid; 1,3-diketones, such as acetylacetone, trifluoroacetylacetone, and thenoyltrifluoroacetone; hydroxycarboxylic acids, such as tartaric acid, citric acid, gluconic acid, and 5-sulfosalicylic acid; polyamines, such as ethylenediamine, diethylenetriamine, triethylenetetramine, and triaminotriethylamine; aminoalcohols, such as triethanolamine and N-(2-hydroxyethyl)ethylenediamine; aromatic heterocyclic bases, such as 2,2'-dipyridyl, 2,2'-diimidazole, dipicoline amine and 1,10-phenanthroline; phenols, such as salicylaldehyde, disulfopyrocatechol, and chromotropic acid; aminophenols, such as 8-hydroxyquinoline and oximesulfonic acid; oximes, such as dimethylglyoxime and salicylaldoxime; peptides containing proximal chelating functionality such as polycysteine, polyhistidine, polyaspartic acid, polyglutamic acid, or combinations of such amino acids; Schiff bases, such as disalicylaldehyde 1,2-propylenediimine; tetrapyrroles, such as tetraphenylporphin and phthalocyanine; sulfur compounds, such as toluenedithiol, meso-2,3-dimercaptosuccinic acid, dimercaptopropanol, thioglycolic acid, potassium

ethyl xanthate, sodium diethyldithiocarbamate, dithizone, diethyl dithiophosphoric acid, and thiourea; synthetic macrocylic compounds, such as dibenzo[18]crown-6, $(CH_3)_6$-[14]-4,11-diene-N4, and (2.2.2-cryptate); and phosphonic acids, such as nitrilotrimethylene-phosphonic acid, ethylenediaminetetra(methylenephosphonic acid), and hydroxyethylidenediphosphonic acid, or combinations of two or more of the above agents.

Preferred residues of chelating agents contain polycarboxylic acid groups and include: ethylenediamine-N,N N',N'-tetraacetic acid (EDTA); N,N,N',N",N"-diethylene-triaminepentaacetic acid (DTPA); 1,4,7,10-tetraazacyclododecane-N,N',N",N"'-tetraacetic acid (DOTA); 1,4,7,10-tetraazacyclododecane-N,N',N"-triacetic acid (DO3A); 1-oxa-4,7,10-triazacyclododecane-N,N',N"-triacetic acid (OTTA); and trans(1,2)-cyclohexanodiethylenetriamine pentaacetic acid (CDTPA).

Preferred residues of chelating agents contain polycarboxylic acid groups and include: B4A, P4A, TMT, DCDTPA, PheMT, macroPheMT, and macroTMT;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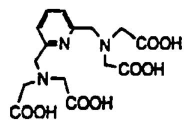
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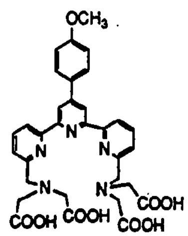
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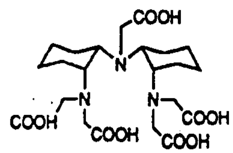
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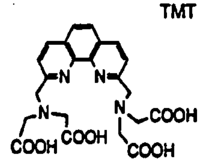
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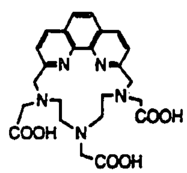
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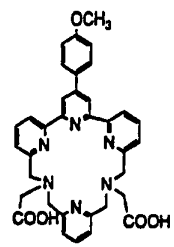
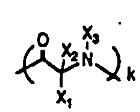
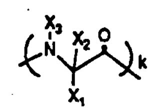

MacroTMT

In one aspect, other suitable residues of chelating agents are comprised of proteins modified for the chelation of metals such as technetium and rhenium as described in U.S. Patent No. 5,078,985, the disclosure of which is hereby incorporated by reference.

In another aspect, suitable residues of chelating agents are derived from N_3S and N_2S_2 containing compounds, as for example, those disclosed in U.S. Patent Nos. 4,444,690; 4,670,545; 4,673,562; 4,897,255; 4,965,392; 4,980,147; 4,988,496; 5,021,556 and 5,075,099.

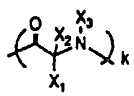
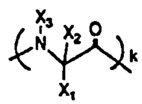
Other suitable residues of chelating agents are described in PCT/US91/08253, the disclosure of which is hereby incorporated by reference. If Q is comprised of the residue of multiple chelating agents, such agents can be linked together by one or more linking groups such as described above.

The residues of the chelating agent Q are independently linked to the other components of this invention through a chemical bond or a linking group such as L_2 as described above. Preferred linking groups also include nitrogen atoms in groups such as amino, imido, nitrilo and imino groups; alkylene, preferably containing from 1 to 18 carbon atoms such as methylene, ethylene, propylene, butylene and hexylene, such alkylene optionally being interrupted by 1 or more heteroatoms such as oxygen, nitrogen and sulfur or heteroatom-containing groups; carbonyl; sulfonyl; sulfinyl; ether; thioether; ester, i.e., carbonyloxy and oxycarbonyl; thioester, i.e., carbonylthio, thiocarbonyl, thiocarbonyloxy, and oxythiocarboxy; amide, i.e., iminocarbonyl and carbonylimino; thioamide, i.e., iminothiocarbonyl and thiocarbonylimino; thio; dithio; phosphate; phosphonate; urelene; thiourelene; urethane, i.e., iminocarbonyloxy, and oxycarbonylimino; an amino acid linkage, i.e., a

 or 

group wherein k=1 and X_1, X_2, X_3 independently are H, alkyl, containing from 1 to 18, preferably 1 to 6 carbon atoms, such as methyl, ethyl and propyl, such alkyl optionally being interrupted by 1 or more heteroatoms such as oxygen, nitrogen and sulfur, substituted or unsubstituted aryl, containing from 6 to 18, preferably 6 to 10 carbon atoms such as phenyl, hydroxyiodophenyl,

hydroxyphenyl, fluorophenyl and naphthyl, aralkyl, preferably containing from 7 to 12 carbon atoms, such as benzyl, heterocyclyl, preferably containing from 5 to 7 nuclear carbon and one or more heteroatoms such as S, N, P or O, examples of preferred heterocyclyl groups being pyridyl, quinolyl, imidazolyl and thienyl; heterocyclylalkyl, the heterocyclyl and alkyl portions of which preferably are described above; or a peptide linkage, i.e., a

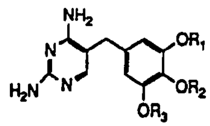
 or 

group wherein k>1 and each X independently is represented by a group as described for X_1, X_2, X_3 above. Two or more linking groups can be used, such as, for example, alkyleneimino and iminoalkylene. It is contemplated that other linking groups may be suitable for use herein, such as linking groups commonly used in protein heterobifunctional and homobifunctional conjugation and crosslinking chemistry as described for L_1 or L_2 above. Especially preferred linking groups include amino groups which when linked to the residue of a chelating agent via an isothiocyanate group on the chelating agent form thiourea groups.

The linking groups can contain various substituents which do not interfere with the coupling reaction between the chelating agent Q and the other components of this invention. The linking groups can also contain substituents which can otherwise interfere with such reaction, but which during the coupling reaction, are prevented from so doing with suitable protecting groups commonly known in the art and which substituents are regenerated after the coupling reaction by suitable deprotection. The linking groups can also contain

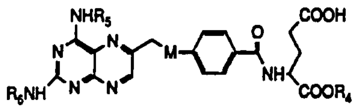
substituents that are introduced after the coupling reaction. For example, the linking group can be substituted with substituents such as halogen, such as F, Cl, Br or I; an ester group; an amide group; alkyl, preferably containing from 1 to about 18, more preferably, 1 to 4 carbon atoms such as methyl, ethyl, propyl, i-propyl, butyl, and the like; substituted or unsubstituted aryl, preferably containing from 6 to about 20, more preferably 6 to 10 carbon atoms such as phenyl, naphthyl, hydroxyphenyl, iodophenyl, hydroxyiodophenyl, fluorophenyl and methoxyphenyl; substituted or unsubstituted aralkyl, preferably containing from 7 to about 12 carbon atoms, such as benzyl and phenylethyl; alkoxy, the alkyl portion of which preferably contains from 1 to 18 carbon atoms as described for alkyl above; alkoxyaralkyl, such as ethoxybenzyl; substituted or unsubstituted heterocyclyl, preferably containing from 5 to 7 nuclear carbon and heteroatoms such as S, N, P or O, examples of preferred heterocyclyl groups being pyridyl, quinolyl, imidazolyl and thienyl; a carboxyl group; a carboxyalkyl group, the alkyl portion of which preferably contains from 1 to 8 carbon atoms; or the residue of a chelating group.

Residues of DHFR active site binding derivatives, trimethoprim analogs, and methotrexate analogs:

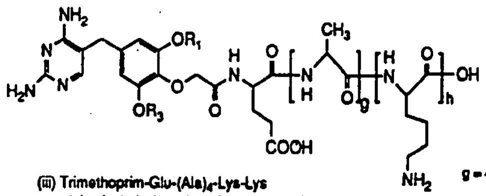


R_1, R_2, and R_3 are independently selected from H, or lower alkyl, aryl, alalkyl, or alkylene-carboxylic acid or amide derivatives.

(i) Trimethoprim analogs

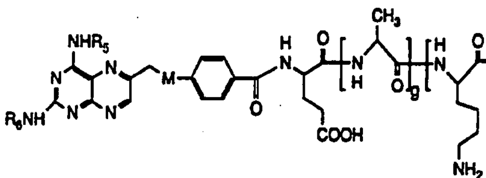


(ii) Methotrexate analogs R_5, R_6 = H, R_4 = H or lower alkyl,
M=N-CH_3, CH_2, O, S.



g = 4; h = 2

(iii) Trimethoprim-Glu-(Ala)$_4$-Lys-Lys
(also include 3'-carbonylmethoxy analogs)

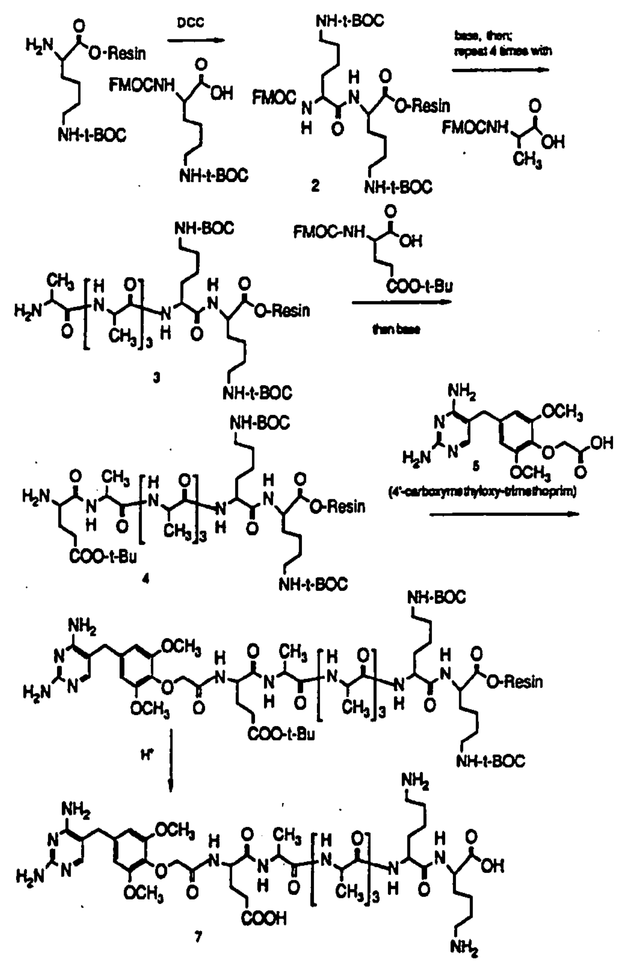


g = 4; h = 2

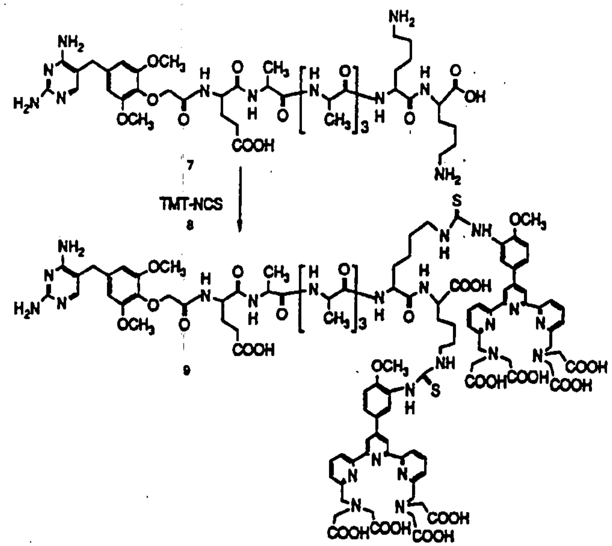
(iv) Methotrexate-Glu-(Ala)$_4$-Lys-Lys

M=N-CH_3, CH_2, O, S. R_5, R_6 = independently H or lower alkyl

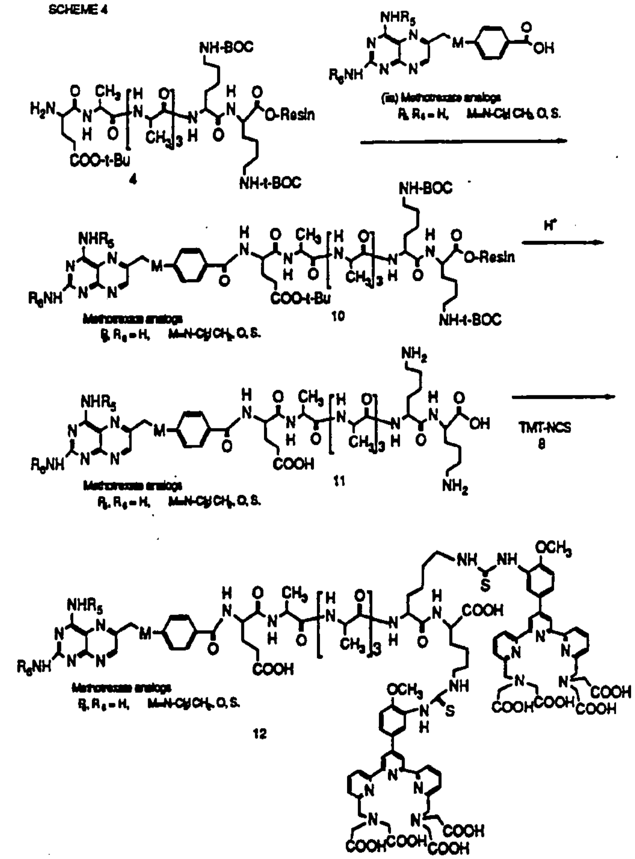
SCHEME 2



SCHEME 3



SCHEME 4



In Systems A and B, specific examples of ligands that have an affinity for non-covalent binding to a DHFR active site useful in this invention include residues of trimethoprim analogs and methotrexate analogs listed in Rahman et al, Methotrexate and Its Analogs; Medicinal Research Reviews, Vol 8, No. 1, 95-157, and Kuyper et al, Carboxysubstituted Trimethoprim Analogs; J. Med. Chem. Vol 28, 303, (1985). Preferred analogs include those defined in structure (i) and in structure (ii), each comprising a reactive group to permit or facilitate binding of one portion of the ligand to the immunoreactive species, Z, in System B and to the chelating agent, Q, in System A.

Ligands comprised of trimethoprim analogs that are useful in the practice of this invention include derivatives defined in structure (i) above where: R_1, R_2, and R_3 are independently selected from H, alkyl (such as methyl as a preferred group for one or two of R_1, R_2, and R_3), aralkyl, aryl (including substituted aryl), an alkylene carboxylic acid or amide derivative thereof, an alkylene group that contains one or more heteroatoms (such as, but not limited to, for example, oxygen [as an ether or a hydroxyl group] or sulfur [as a thioether group]), an amino acid group and a peptide group, and at least one of $-R_1$, $-R_2$, and $-R_3$ is of the form represented in structure 13:

$$(X)_x - L_v - \left[[A]_a - \underset{\substack{| \\ Q_q \\ | \\ M}}{[B]_b} \right]_f - W \qquad 13$$

wherein:
X is an alkylene group from 1 to 12 carbon atoms which may contain one or more heteroatoms such as oxygen, sulfur or nitrogen; L is a linking group as defined above, preferably the residue of an amide group, a chemical bond, an amino acid residue, or an arylene group which may be substituted by one or more hydroxyl groups; A is an alkylene group, a polyalkylene oxidyl group, an amino acid residue, a peptide residue, X, or a group containing pendant substituents which contain heteroatoms (such as, for example, oxygen in the form of one or more hydroxyl groups, carboxylic acid groups or salts thereof, amido groups, ether groups, sulfur in the form of thioether, sulfone, sulfoxide or sulfonate, nitrogen in the form of amino groups, amido groups or a diazo linkage, or phosphorous in the form of phosphate); B is selected from A but modified to contain one or more radionuclides bound thereto by chelating groups, Q, as defined above, such as, for example, but not limited to, TMT groups or DTPA groups, macrocyclic chelating groups, chelate groups that contain sulfur atoms, chelate groups that contain nitrogen atoms, chelate groups that contain pyridine rings, and chelate groups that contain carboxylate or phosphate groups; W is selected from H, alkyl, aralkyl, alkylene, carboxylic acid groups, amino groups, amido groups, aryl groups, hydroxyaryl groups, a therapeutically effective and diagnostically effective radioisotope of an atom (such as iodine and the like) that can be covalently attached to a component of structure (i) [as distinct from a therapeutically effective or diagnostically effective radioisotope of an ion that can be bound to a component of structure (i) via a chelate group], a chelate group that may contain a therapeutically effective or diagnostically effective radioisotope, M, of a metal ion such as yttrium, indium, rhenium, copper, scandium, bismuth, lead, or leuticum

and the like; x and v are independently zero or one; a is zero or an integer from one to about 100; b, q, and f are independently integers from one to about 100.

In the RDA of System A, a preferred non-limiting example of a linking group includes the residue of a protected (D)-Glu-$(Ala)_4$-Lys-Lys (4) as well as the residue of a protected (L)-Glu-$(Ala)_4$-$(Lys)_2$. Both 4'-carboxymethyloxy-trimethoprim (structure 5) and methotrexate analog (structure iia) undergo coupling reactions (see SCHEMES 2, 3 and 4) followed by deprotection to afford trimethoprim-(D)-Glu-$(Ala)_4$-Lys-Lys (structure iii) and methotrexate (structure iv) and the analogous L-Glu containing species, respectively. The synthetic schemes (2,3,4) describe the synthetic methods for trimethoprim-heptapeptide (structure iii) and methotrexate-heptapeptide (structure iv). Thus, the t-butoxycarbonyl (tBoc) blocked Lys-Lys (structure 2) is prepared using a dehydrative coupling method, for example, using DCC (dicyclohexylcarbodiimide) and two protected lysine groups (SCHEME 2). The FMOC (9-Fluorenylmethoxycarbonyl) group of Lys-Lys is then removed via base treatment, and 4 units of alanine are introduced as shown in SCHEME 2 to afford the blocked Ala-Ala-Ala-Ala-Lys-Lys (structure 3). After adding t-butyl (tBu) blocked D-Glu on the N-terminal of the hexapeptide (structure 3), the resulting heptapeptide (structure 4) is coupled with 4'-carboxymethyloxy-trimethoprim (structure 5) to yield trimethoprim-heptapeptide (structure 6). The acidic deblocking of the peptide (structure 6) affords the desired trimethoprim-D-Glu-$(Ala)_4$-Lys-Lys (structure 7). trimethoprim-D-Glu-$(Ala)_4$-Lys-Lys (structure 7) upon treatment with TMT-NCS (structure 8) (SCHEME 3) yields a desirable ligand-to-chelating agent conjugate, trimethoprim-D-Glu-$(Ala)_4$-[Lys-(TMT)]-[Lys-(TMT)], (structure 9). A solution of trimethoprim-D-Glu-$(Ala)_4$-

[Lys-(TMT)]-[Lys-(TMT)] (structure 9) in deionized water buffered with 0.5 M sodium acetate at a pH of about 6.0 at room temperature is treated with an aqueous HCl solution of $^{90}YCl_3$ to afford a radionuclide labeled (^{90}Y) trimethoprim-D-Glu-$(Ala)_4$-[Lys-(TMT)]-[Lys-(TMT)].

Employing the similar synthetic methods, but using a blocked L-Glu derivative, the analogous L-Glu-trimethoprim derived materials are obtained.

Employing the similar synthetic methods, a coupling reaction (Scheme 4) of the heptapeptide (structure 4) and the benzoic acid derivative, 4'-carboxy-methotrexate (structure iia), yields the desired blocked methotrexate-D-Glu-$(Ala)_4$-Lys-Lys (structure 10). methotrexate-heptapeptide (structure 11), is obtained from acid deblocking of (structure 10), and reacts with TMT-NCS to afford the desirable methotrexate-D-Glu-$(Ala)_4$-[Lys-(TMT)]-[Lys-(TMT)] (structure 12). A solution of methotrexate-D-Glu-$(Ala)_4$-[Lys-(TMT)]-[Lys-(TMT)] (structure 12) in deionized water buffered with 0.5 M sodium acetate at a pH of about 6.0 at room temperature is treated with an aqueous HCl solution of $^{90}YCl_3$ to afford a radionuclide labeled (^{90}Y) methotrexate-D-Glu-$(Ala)_4$-[Lys-(TMT)]-[Lys-(TMT)].

Employing the similar synthetic methods, but using a blocked L-Glu derivative, the analogous L-Glu-methotrexate derived materials are obtained.

Racemic mixtures of D and L enantiomers of the above described trimethoprim and methotrexate derivatives are also useful in this invention.

Additional chelating agents and radionuclides bound to chelating agents are incorporated by preparing, for example, analogous peptides comprised of additional Lys-TMT and Lys-TMT-radionuclide groups. Preferably, the number of such Lys-TMT and Lys-TMT-radionuclide residues is from 1 to about 6, and more preferably from 2 to about 6.

In the System B, the NRTIR is comprised of one or more ligands that have an affinity for non-covalent binding to a DHFR active site each with a suitably substituted linking group (L_1) conjugated to the immunoreactive group (Z). Preferably, said ligand that has an affinity for non-covalent binding to a DHFR active site is comprised of a residue of (structure i) or (structure ii) linked to Z by a linking group L_1 as defined above. The NRTIR preferably contains 2 to about 10 of such groups, more preferably 2 to about 4.

The delivery agent in the System B is comprised of a DHFR active site moiety conjugated to one or more chelating agents via a linking group as described above.

In one embodiment, both in System A and System B, it is desirable that the radionuclide be a metal ion and that said metal ion be easily complexed to the chelating agent, for example, by merely exposing or mixing an aqueous solution of the chelating agent-containing moiety with a metal salt in an aqueous solution preferably having a pH in the range of about 4 to about 11. The salt can be any salt, but preferably the salt is a water soluble salt of the metal such as a halogen salt, and more preferably such salts are selected so as not to interfere with the binding of the metal ion with the chelating agent. The chelating agent-containing moiety is preferrably in aqueous solution at a pH of between about 5 and about 9, more preferably between pH about 6 to about 8. The chelating agent-containing moiety can be mixed with buffer salts such as citrate, acetate, phosphate and borate to produce the optimum pH. Preferably, said buffer salts are selected so as not to interfere with the subsequent binding of the metal ion to the chelating agent.

In therapeutic applications, the RDA of this invention preferably contains a ratio of metal radionuclide ion to chelating agent that is effective in

such therapeutic applications. In preferred embodiments, the mole ratio of metal ion per chelating agent is from about 1:100 to about 1:1.

In diagnostic imaging applications, the RDA of this invention preferably contains a ratio of metal radionuclide ion to chelating agent that is effective in such diagnostic imaging applications. In preferred embodiments, the mole ratio of metal ion per chelating agent is from about 1:1,000 to about 1:1.

In another embodiment, the RDA of this invention can comprise a non-radioisotope of a metal ion. The metal ions can be selected from, but are not limited to, elements of groups IIA through VIA. Preferred metals include those of atomic number 12, 13, 20, the transition elements 21 - 33, 38 - 52, 56, 72 - 84 and 88 and those of the lanthanide series (atomic number 57 - 71).

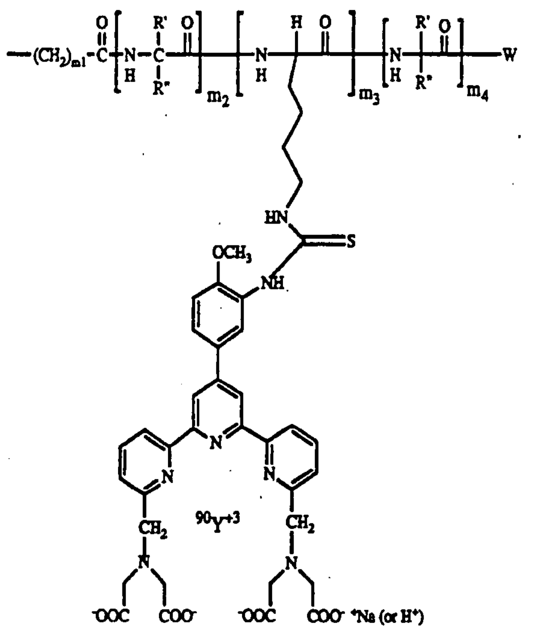
In another embodiment, the RDA of this invention can comprise a radionuclide. The radionuclide can be selected, for example, from radioisotopes of Sc, Fe, Pb, Ga, Y, Bi, Mn, Cu, Cr, Zn, Ge, Mo, Tc, Ru, In, Sn, Sr, Sm, Lu, Sb, W, Re, Po, Ta and Tl. Preferred radionuclides include ^{44}Sc, ^{64}Cu, ^{67}Cu, ^{111}In, ^{212}Pb, ^{68}Ga, ^{90}Y, ^{153}Sm, ^{212}Bi, ^{99m}Tc, ^{186}Re and ^{188}Re. Of these, especially preferred is ^{90}Y. These radioisotopes can be atomic or preferably ionic.

In another embodiment, the RDA of this invention can comprise a fluorescent metal ion. The fluorescent metal ion can be selected from, but is not limited to, metals of atomic number 57 to 71. Ions of the following metals are preferred: La, Ce, Pr, Nd, Pm, Sm, Eu, Gd, Tb, Dy, Ho, Er, Tm, Yb, and Lu. Eu is especially preferred.

In another embodiment, the RDA of this invention can comprise one or more paramagnetic elements which are suitable for the use in MRI applications. The

paramagnetic element can be selected from elements of atomic number 21 to 29, 43, 44 and 57 to 71. The following elements are preferred: Cr, V, Mn, Fe, Co, Ni, Cu, La, Ce, Pr, Nd, Pm, Sm, Eu, Gd, Tb, Dy, Ho, Er, Tm, Yb and Lu. Mn, Gd, and Dy are especially preferred.

An example of a structure of an RDA that has utility in this invention is structure (i) above wherein R_1, R_2, and R_3 are as defined above and preferably wherein one of R_1, R_2, and R_3 is represented by structure 14.



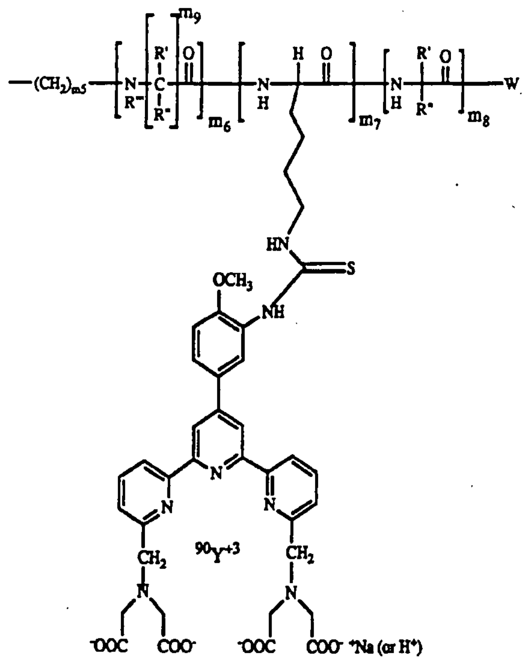
14

where: R' and R" are selected from components of amino acids that comprise, for example, natural amino acids such as glycine, alanine, leucine, serine, lysine, isoleucine, glutamine, aspartic acid, glutamic acid, proline, threonine, valine, phenylalanine, tyrosine, and the like, as well as unnatural amino acids and racemates of natural amino acids, and R' and R" can independently be selected from H, a polyalkylene oxidyl group, and branched peptide groups which may contain additional chelate groups such as TMT;

m_1 is an integer between 1 and 10, m_2, m_3, and m_4 are independently selected from zero and an integer between 1 and 10 with the proviso that m2 is at least 1, and preferably 2 to about 5; and

W is selected from OH, NH_2, a residue of TMT, an O-alkyl group such as an O-methyl group, and NR_aR_b wherein R_aR_b are independently selected from alkyl groups such as methyl groups, H, and a polyalkylene oxidyl moiety such as PEG-OH and PEG-O-alkyl (e.g., PEG-O-CH_3) with the PEG having an average molecular weight in the range between 45 and 5,000 daltons.

Another example of a structure that has utility in this invention is structure (i) above wherein R_1, R_2, and R_3 are as defined above and preferably wherein one of R_1, R_2, or R_3 is represented by structure 15.



15

wherein: R' and R" are selected from components of amino acids that comprise, for example, natural amino acids such as glycine, alanine, leucine, serine, lysine, isoleucine, glutamine, aspartic acid, glutamic acid, proline, threonine, valine, phenylalanine, tyrosine, and the like, as well as unnatural amino acids or racemates of natural amino acids, and R' and R" can be independently selected from H, a polyalkylene oxidyl group, a branched peptide group which may contain one to about 10 additional chelate groups such as TMT;
m_5 is an integer between 1 and 10, m_6, m_7, and m_8 are independently selected from zero and an integer between

1 and 10 with the proviso that m_7 is at least 1, and preferably 2 to about 5;
W is selected from OH, NH_2, a TMT moiety, an O-alkyl group such as an O-methyl group, NR_aR_b wherein R_aR_b are independently selected from alkyl groups such as methyl, H, a polyalkylene oxide moiety such as PEG-OH and PEG-O-alkyl (e.g., PEG-O-CH_3) with the PEG having molecular weight in the range between 45 and 5,000 daltons;
m_9 is selected from zero and an integer from 1 to about 12; and
R''' is selected from H and the residue of a TMT moiety with or without a radionuclide, said TMT moiety being linked via a thiourea group.

In another embodiment of this invention, an RDA comprised of at least two metal ions in combination with one another in the same formulation is specifically contemplated. For example, the use of a therapeutically effective dose of a radionuclide such as $^{90}Y^{+3}$ together with a diagnostic imaging effective dose of a paramagnetic ion such as Gd^{+3}, the ratio of the molar concentration of the diagnostic imaging effective ion to the molar concentration of the radionuclide ion being typically greater than one, in a pharmaceutically effective formulation of said RDA permits the simultaneous magnetic resonance imaging of at least a portion of the tissue of a host patient during therapeutic treatment of said patient.

In another embodiment of this invention, the use of radioisotopes of iodine is specifically contemplated. For example, if the RDA of System A or of System B is comprised of substituents that can be chemically substituted by iodine in a covalent bond forming reaction, such as, for example, substituents containing hydroxyphenyl functionality, such substituents can be labeled by methods well known in the art with a radioisotope of iodine. The thus covalently linked

iodine species can be used in the aforementioned fashion in therapeutic and diagnostic imaging applications.

In a preferred embodiment, an effective dose of an RDA of System A or of System B as described above in a pharmaceutically acceptable medium is prepared by exposing a composition of a precursor of an RDA (said precursor being comprised of a residue of a ligand that has an affinity for non-covalent binding to a DHFR active site, a linking group, and a residue of a chelating agent in System A and of a residue of a DHFR active site, a linking group, and a residue of a chelating agent in System B) to a composition containing a radioactive metal ion such that the molar amount of said radionuclide metal ion is less than the molar amount of the chelating groups comprising the RDA in said composition, said duration of exposure lasting an effective time to permit uptake of said metal ion into said RDA.

In a preferred embodiment, an effective dose of a NRTIR of System A or System B as described above in a pharmaceutically acceptable medium is administered to a patient and said NRTIR is allowed to accumulate at the target site such as at a tumor site in said patient. Subsequently, at an effective time, an effective dose of a RDA as described above in a pharmaceutically acceptable medium is administered to said patient, and said RDA is allowed to accumulate at the target site, said target site being the said NRTIR accumulated at said tumor site in said patient.

In a preferred embodiment, a therapeutically effective dose of a NRTIR of System A or System B as described above in a pharmaceutically acceptable medium is administered to a patient or to a tissue from a patient and said NRTIR is allowed to accumulate at the target site such as at a tumor site in said patient. Subsequently, at a therapeutically effective time, a

therapeutically effective dose of a RDA as described above in a pharmaceutically acceptable medium is administered to said patient, and said RDA is allowed to accumulate at the target site, said target site being the said NRTIR accumulated at said tumor site in said patient.

The present invention also comprises one or more NRTIR as described above formulated into compositions together with one or more non-toxic physiologically acceptable carriers, adjuvants or vehicles which are collectively referred to herein as carriers, for parenteral injection for oral administration in solid or liquid form, for rectal or topical administration, or the like. The present invention also comprises one or more RDA as described above formulated into compositions together with one or more non-toxic physiologically acceptable carriers, adjuvants or vehicles which are collectively referred to herein as carriers, for parenteral injection, for oral administration in solid or liquid form, for rectal, or topical administration, or the like.

The compositions can be administered to humans and animals either orally, rectally, parenterally (intravenous, by intramuscularly or subcutaneously), intracisternally, intravaginally, intraperitoneally, intravesically, locally (powders, ointments or drops), or as a buccal or nasal spray. It is specifically contemplated that the NRTIR and the RDA can be administered by the same route such as orally, rectally, parenterally (intravenous, by intramuscularly or subcutaneously), intracisternally, intravaginally, intraperitoneally, intravesically, locally (powders, ointments or drops), or as a buccal or nasal spray. It is also contemplated that the NRTIR can be administered by a route different from that of the RDA.

Compositions suitable for parenteral injection may comprise physiologically acceptable sterile aqueous or nonaqueous solutions, dispersions, suspensions or emulsions and sterile powders for reconstitution into sterile injectable solutions or dispersions. Examples of suitable aqueous and nonaqueous carriers, diluents, solvents or vehicles include water, ethanol, polyols (propyleneglycol, polyethyleneglycol, glycerol, and the like), suitable mixtures thereof, vegetable oils (such as olive oil) and injectable organic esters such as ethyl oleate. Proper fluidity can be maintained, for example, by the use of a coating such as lecithin, by the maintenance of the required particle size in the case of dispersions and by the use of surfactants.

These compositions may also contain adjuvants such as preserving, wetting, emulsifying, and dispensing agents. Prevention of the action of microorganisms can be ensured by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, sorbic acid, and the like. It may also be desirable to include isotonic agents, for example sugars, sodium chloride and the like. Prolonged absorption of the injectable pharmaceutical form can be brought about by the use of agents delaying absorption, for example, aluminum monostearate and gelatin.

Solid dosage forms for oral administration include capsules, tablets, pills, powders and granules. In such solid dosage forms, the active compound is admixed with at least one inert customary excipient (or carrier) such as sodium citrate or dicalcium phosphate or (a) fillers or extenders, as for example, starches, lactose, sucrose, glucose, mannitol and silicic acid, (b) binders, as for example, carboxymethylcellulose, alignates, gelatin, polyvinylpyrrolidone, sucrose and acacia, (c) humectants, as for example, glylcerol, (d) disintegrating agents, as for example, agar-agar,

calcium carbonate, potato or tapioca starch, alginic acid, certain complex silicates and sodium carbonate, (e) solution retarders, as for example paraffin, (f) absorption accelerators, as for example, quaternary ammonium compounds, (g) wetting agents, as for example, cetyl alcohol and glycerol monostearate, (h) adsorbents, as for example, kaolin and bentonite, and (i) lubricants, as for example, talc, calcium stearate, magnesium stearate, solid polyethylene glycols, sodium lauryl sulfate or mixtures thereof. In the case of capsules, tablets and pills, the dosage forms may also comprise buffering agents.

Solid compositions of a similar type may also be employed as fillers in soft and hard-filled gelatin capsules using such excipients as lactose or milk sugar as well as high molecular weight polyethyleneglycols, and the like. Solid dosage forms such as tablets, dragees, capsules, pills and granules can be prepared with coatings and shells, such as enteric coatings and others well known in the art. They may contain opacifying agents, and can also be of such composition that they release the active compound or compounds in a certain part of the intestinal tract in a delayed manner. Examples of embedding compositions which can be used are polymeric substances and waxes.

The active compounds can also be in micro-encapsulated form, if appropriate, with one or more of the above-mentioned excipients.

Liquid dosage forms for oral administration include pharmaceutically acceptable emulsions, solutions, suspensions, syrups and elixirs. In addition to the active compounds, the liquid dosage forms may contain inert diluents commonly used in the art, such as water or other solvents, solubilizing agents and emulsifiers, as for example, ethyl alcohol, isopropyl alcohol, ethyl carbonate, ethyl acetate, benzyl alcohol, benzyl

benzoate, propyleneglycol, 1,3-butyleneglycol, dimethylformamide, oils, in particular, cottonseed oil, groundnut oil, corn germ oil, olive oil, castor oil and sesame oil, glycerol, tetrahydrofurfuryl alcohol, polyethyleneglycols and fatty acid esters of sorbitan or mixtures of these substances, and the like.

Besides such inert diluents, the composition can also include adjuvants, such as wetting agents, emulsifying and suspending agents, sweetening, flavoring and perfuming agents.

Suspensions, in addition to the active compounds, may contain suspending agents, as for example, ethoxylated isostearyl alcohols, polyoxyethylene sorbitol and sorbitan esters, microcrystalline cellulose, aluminum metahydroxide, bentonite, agar-agar and tragacanth, or mixtures of these substances, and the like.

Compositions for rectal administrations are preferably suppositories which can be prepared by mixing the compounds of the present invention with suitable non-irritating excipients or carriers such as cocoa butter, polyethyleneglycol or a suppository wax, which are solid at ordinary temperatures but liquid at body temperature and therefore, melt in the rectum or vaginal cavity and release the active component.

Dosage forms for topical administration of a compound of this invention include ointments, powders, sprays and inhalants. The active component is admixed under sterile conditions with a physiologically acceptable carrier and any preservatives, buffers or propellants as may be required. Opthalmic formulations, eye ointments, powders and solutions are also contemplated as being within the scope of this invention.

Actual dosage levels of active ingredients in the compositions of the present invention may be varied so

as to obtain an amount of active ingredient that is effective to obtain a desired therapeutic response for a particular composition and method of administration. The selected dosage level therefore depends upon the desired therapeutic effect, on the route of administration, on the desired duration of treatment and other factors.

The total daily dose of the compounds of this invention administered to a host in single of divided dose may be in amounts, for example, of from about 1 nanomol to about 5 micromols per kilogram of body weight. Dosage unit compositions may contain such amounts of such submultiples thereof as may be used to make up the daily dose. It will be understood, however, that the specific dose level for any particular patient will depend upon a variety of factors including the body weight, general health, sex, diet, time and route of administration, rates of absorption and excretion, combination with other drugs and the severity of the particular disease being treated.

In another embodiment, the present invention is directed to a method of diagnosis comprising the administration of a diagnostic imaging effective amount of the compositions of the present invention to a mammal or to a tissue from said mammal in need of such diagnosis. A method for diagnostic imaging for use in medical procedures in accordance with this invention comprises administering to the body of a test subject in need of a diagnostic image an effective diagnostic image producing amount of the above-described compositions.

In this method, an effective diagnostic image producing amount of a non-radioactive targeting immunoreagent (NRTIR) as described above in a pharmaceutically acceptable medium is administered to a patient and said non-radioactive targeting immunoreagent is allowed to accumulate at the target site such as at a

tumor site in said patient. Subsequently, a diagnostic imaging effective dose of a radioactive delivery reagent (RDA) as described above in a pharmaceutically acceptable medium is administered to said patient, and said radioactive targeting reagent is allowed to accumulate at the target site, said target site being the said non-radioactive targeting immunoreagent accumulated at said tumor site in said patient. The image pattern can then be visualized.

Alternatively, the NRTIR may be reacted with a diagnostic imaging effective amount of a reagent comprised of a radionuclide prior to administration to the environs of a tissue of interest of a patient undergoing such diagnostic imaging, waiting for an effective period of time during which time said NRTIR will bind to sites on cells of said tissue of interest and during which time unbound NRTIR will be removed from the environs of said tissue and then obtaining an image as a function of time of all or part of said tissue of interest. When the image of all or part of said tissue of interest is optimal, a diagnostic imaging or a therapeutically effective amount of RDA containing the same or a different radionuclide as that employed on the NRTIR is administered to said tissue of interest of said patient.

In addition to human patients, the test subjects can include mammalian species such as rabbits, dogs, cats, monkeys, sheep, pigs, horses, bovine animals and the like.

After administration of the compositions of the present invention, the subject mammal is maintained for a time period sufficient for the administered compositions to be distributed throughout the subject and enter the tissues of the mammal. A sufficient time period is generally from about 1 hour to about 2 weeks

or more and, preferably from about 2 hours to about 1 week.

The following examples further illustrate the invention and are not to be construed as limiting of the specification and claims in any way. Specific embodiments of the invention are illustrated in the following examples.

EXAMPLES

Example 1

5-(4'Hydroxy-3',5'-dimethoxybenzyl)-2,4-diaminopyrimidine

To a solution of 22 g (0.2 mol) of 2,4-diaminopyrimidine and 49.5 g (0.2 mol) of 2,6-dimethoxy-4-[(N,N-dimethylamino)methyl]phenol hydrochloride in 300 ml of ethylene glycol was added 11 g (0.203 mol) of sodium methoxide. The reaction mixture was heated under nitrogen with stirring to 150-160°C, at which point dimethylamine was evolved. After 3 hours, 80% of the theoretical amount of dimethylamine was collected. The solvent was removed in vacuo (at 85°C), the residual oil was washed with water, and then with acetone. The product was obtained as a tan precipitate (yield 25 g).

Example 2

5-(4'-Methoxycarbonylmethyloxy-3',5'-dimethoxybenzyl)-2,4-diaminopyrimidine

5-(4'-Hydroxy-3',5'-dimethoxybenzyl)-2,4-diaminopyrimidine (5.52 g; 20 mmol) in 80 ml of DMSO was allowed to react with t-BuOH (2.469 g; 22 mmol) and methyl bromoacetate (3.366 g; 22 mM) for 14 hours. The

solvent was removed in vacuo, and the residual solid was vigrously shaken with saturated sodium bicarbonate solution. A light brown solid was filtered and dried to yield 1.5 g of the desired ester, m.p. 149-151°C. A second crop (3 g) of less pure material was also isolated. These two were combined for hydrolysis in Example 3.

Example 3

<u>5-(4'-Carboxymethyloxy-3',5'-dimethoxybenzyl)-2,4-diaminopyrimidine, trimethoprim-4'-O-Acetic Acid</u>

5-(4'-Methoxycarbonylmethyloxy-3',5'-dimethoxybenzyl)-2,4-diaminopyrimidine (4.5 g; 15.48 mM) was dissolved in 50 ml of 2 M NaOH. The clear solution was heated at 60°C for 4 hours, cooled, and allowed to stand overnight. A white precipitate formed which was filtered, washed with acetone, then with ether, and then dried. Yield: 20 g; melting point 274°C. H nmr (DMSO-d6): d 7.5 (S, 1H), 6.53 (S, 2H), 6.08 (S, 2H), 5.69 (S, 2H), 3.9 (S, 2H), 3.7 (S, 6H) and 3.50 ppm (S, 2H). Mass spectrum (CI): MH^+ = 357 (Na+ salt).

<u>Preparation of H_2N-[D-Glu]=Ala-Ala-Ala-Ala-Lys-Lys-OH</u>

The linear peptide H_2N-[D-Glu]-Ala-Ala-Ala-Ala-Lys-Lys-OH was synthesized via solid-phase methodology, on an ABI 430A Automated Peptide Synthesizer. The solid support used in the synthesis was a 4-Alkoxybenzyl alcohol polystyrene resin (Wang resin). The N-alpha-Fmoc protecting group was used throughout the synthesis, with t-butyl side chain protection on D-Glu, and t-BOC protection on the side chain of Lys. The peptide chain

was assembled using the ABI FastMoc™ software protocols (0.25 mmole scale, HBTU activated couplings, 4 fold excess of amino acid, 1 hour) for Fmoc-chemistry.

<u>Preparation of trimethoprim-4'-O-acetic acid amide of H_2N-[D-Glu]-Ala-Ala-Ala-Ala-Lys-Lys-OH</u>

Addition of trimethoprim-4'-O-acetic acid of Example 3 to the N-terminus of the above peptide-resin was carried out manually by adding in order: 335 mg trimethoprim (1 mmole) in 25 ml of DMSO, 525 uL of diisopropylethylamine (3 mmole) followed by 380 mg of HBTU (1 mmole). The mixture was allowed to react for 2 hours at room temperature, at which time the mixture was filtered, and the peptide resin washed 3 x 50 ml DMF and then by 3 x 50 ml MeOH.

Removal and deprotection of the peptide was accomplished by adding 15 ml of a 95:5 TFA/H2O solution to the peptide-resin, in a sealed vessel and shaken at room temperature for 2 hours. At the end of 2 hours, the mixture was filtered by pouring into a scintered glass funnel. The filtrate volume was then reduced to an oil (@ 3 ml) by rotoevaporation. The peptide was then precipitated by dropping the oil into a centrifuge tube containing 50 ml of Et_2O. The peptide was spun down and the ether decanted, and the peptide was allowed to air dry.

Example 4

<u>Preparation of radionuclide labeled (^{90}Y)-trimethoprim-D-Glu-$(Ala)_4$-Lys-(TMT)-Lys-(TMT) (Scheme 1 & 2)</u>

To a mixture of resin containing D-Glu-(COOtBu)-$(Ala)_4$-Lys-tBOC-Lys-tBoc (4; 30mM) and DCC in 150 ml of methylene chloride is added in portions a solution of 5-

(4'-carboxymethyloxy-3',5'-dimethoxybenzyl)-2,4-diaminopyrimidine (i.e., trimethoprim-4'-O-acetic acid) (5; 30mM) in 100 ml of methylene chloride. The resulting reaction mixture is stirred 2 days, and the solvent and the urea are removed to afford the blocked trimethoprim-4'-O-acetic acid heptapeptide (6), which upon mild hydrolysis with trifluoroacetic acid, yields trimethoprim-4'-O-acetic acid heptapeptide (7).

A solution of trimethoprim-4'-O-acetic acid heptapeptide (7) (20 mM) in 50 ml of a saturated aqueous sodium bicarbonate at about pH 9 is allowed to react with TMT-isothiocyanate (8; 20mM) at room temperature for 12 hours to afford trimethoprim-4'-O-acetic acid-D-Glu-(ALA)$_4$-[Lys-(TMT)]-[Lys-(TMT)] (9).

A solution of the above trimethoprim-4'-O-acetic acid-D-Glu-(ALA)$_4$-[Lys-(TMT)]-[Lys-(TMT)] (9) in deionized water buffered with 0.5 M sodium acetate at pH 6.0 at room temperature is treated with a solution of $^{90}YCl_3$ in aqueous hydrochloric acid. Uptake of the radiolabel into the chelator is demonstrated by thin layer chromatography. In excess of 97% of the added ^{90}Y is associated with trimethoprim-4'-O-acetic acid-D-Glu-(ALA)$_4$-[Lys-(TMT)]-[Lys-(TMT)] to form the desired ^{90}Y-labelled product.

Example 5

<u>Preparation of radionuclide labeled (^{90}Y)-methotrexate-D-Glu-(Ala)$_4$-Lys-(TMT)-Lys-(TMT) (Scheme 3)</u>

To a mixture of resin containing D-Glu-(COOtBu)-(Ala)$_4$-(Lys-tBOC)-(Lys-tBoc) (4; 30mM) and DCC in 150 ml of methylene chloride is added in portions a solution of 4'-carboxy-methotrexate (iia; 30mM) in 100 ml of methylene chloride. The resulting reaction mixture is stirred 2 days, and the solvent and the urea are removed

to afford the blocked methotrexate-heptapeptide (10), which upon mild hydrolysis with 4 N HCl, yields 4-carboxymethotrexate-heptapeptide (11).

To a solution of 4-carboxymethotrexate-heptapeptide (11; 20 mM) in 50 ml of methylene chloride is allowed to react with TMT-isothiocyanate (8; 20mM) at room temperature for 12 hours to afford 4'-carboxymethotrexate-D-Glu-(ALA)$_4$-[Lys-(TMT)]-[Lys-(TMT)] (12).

A volume of radioactive Yttrium chloride (^{90}Y in 0.04M hydrochloric acid at a specific activity of >500 Ci/mg: Amersham-Mediphysics) is neutralized using two volumes of 0.5 M sodium acetate pH 6.0 and added to a solution of the above 4'-carboxymethotrexate-D-Glu-(ALA)$_4$-[Lys-(TMT)]-[Lys-(TMT)] (12) in deionized water buffered with 0.5 M sodium acetate at pH 6.0 at room temperature. The labeling is allowed to proceed for one hour and then the labeling efficiency is determined by thin layer chromatography on a Gelman ITAL-SG strip developed in 0.1 M sodium citrate, pH 6.0. In excess of 97% of the added ^{90}Y is taken up by the 4'-carboxymethotrexate-D-Glu-(Ala)$_4$-[Lys-(TMT)]-[Lys-(TMT)] to form the desired ^{90}Y-labeled product.

The following examples illustrate the construction of conjugates between an antibody and dihydrofolate reductase (DHFR). In these examples ING-1 (a chimeric IgG_1 antibody) is chosen for the methodologies; other antibodies such as those described herein are useful. The DHFR referred to below is of bacterial origin, produced as a recombinant product from the cloned DHFR gene which is overexpressed in Escherchia coli (E. coli), or of human origin, available as a recombinant protein.

Examples of System A

SYSTEM A

	Non-Radioactive Targeting ImmunoReagent NRTIR	Radioactive Delivery Agent RDA
1	Immunoreactive group + (linking group + receptor)$_n$	Ligand + (chelating agent + radionuclide)$_m$
2	Z-(L_1-Rec)$_n$	D-(L_2-Q-M)$_m$
3	Z-(L_1-Rec)$_n$	Trimethoprim-(L_2-Q-M)$_m$
4	Z-(L_1-Rec)$_n$	Methotrexate-(L_2-Q-M)$_m$

wherein:

Z is the residue of an immunoreactive group;

Rec is the residue of a receptor, preferably DHFR;

D is the residue of a ligand that has an affinity for non-covalent binding to the receptor, preferably to a DHFR receptor;

DHFR ligand is the residue of a ligand that has an affinity for non-covalent binding to a DHFR active site;

TMP is the residue of a trimethoprim analog;

MTX is the residue of a methotrexate analog;

L_1 and L_2 are each independently the residue of a linking group that may independently contain spacing group;

Q is the residue of a chelating group;

M is a radionuclide; and

n and m are each independently an integer greater than zero.

Example 6

(6a) Preparation of Antibody-Maleimide with Sulfo-SMCC (ING-1-Maleimide) [Z-L_1]

A sulfo-SMCC solution (36 nmoles) in PBS was added to a sample of a chimeric antibody (ING-1; 6 nmoles) solution in phosphate buffer (pH7). The resulting mixture was allowed to stand for 30 minutes with occasional mixing at room temperature.The reaction mixture was diluted with phosphate buffered saline, added to a prewashed PD-10 column (Pharmacia), and eluted with PBS to afford ING-1-maleimide. This material was stored on ice until use.

(6b) Preparation of mercaptoalkyl-antibody [Z-L_1]

A sample of of a chimeric antibody (ING-1; 6 nmoles) solution in 0.1 M carbonate buffer (pH 8.8) is mixed with 200 nmoles of an aqueous solution of 2-iminothiolane. The resulting mixture is allowed to stand for 30 minutes with occasional mixing at room temperature. The reaction mixture is diluted with phosphate buffed saline, added to a prewashed PD-10 column, and eluted with PBS to afford mercaptoalkyl-ING-1. This material is stored on ice until use.

(6c) Preparation of mercapto-antibody using SATA

A solution containing 6 nmoles of ING-1 in PBS is vortexed while 60 nmoles of SATA (in DMSO) are added. After mixing and standing at room temperature for 60 minutes, the reaction mixture is diluted with PBS, and eluted from a PD-10 column with PBS to afford ING-1-CO-CH_2-S-CO-CH_3. The acetylthioacetylated antibody is deprotected by the addition of 30 uL of a pH 7.5

solution contaning 100 mM sodium phosphate, 25 mM EDTA, 50 mM NH2OH. The reaction proceeds for two hours at room temperature after which the material is again passed down a PD-10 column by elution with PBS. The final product (ING-1-CO-CH_2-SH) is used immediately.

(6d) Radiolabeling of ING-1 with ^{125}I

An aliquot of ING-1 (500 ug) is labeled with ^{125}I monochloride (at about 5 mCi/mg) in the presence of iodogen (Sodium N-chloro-benzenesulfonamide) beads in a volume of 500 uL 100mm phosphate buffer (pH 7.2) at room temperature. After 15 minutes the reaction is terminated by passage of the labeled antibody down a prewashed NAP-5 column (Pharmacia). The radioiodinated protein is eluted with PBS and stored at 4°C until use.

Example 7

(7a) Preparation of mercapto-DHFR using SATA

A solution containing 50 nmoles of DHFR in PBS is vortexed while 500 nmoles of SATA (in DMSO) are added. After mixing and standing at room temperature for 60 minutes, the reaction mixture is diluted with PBS, and eluted from a PD-10 column with PBS to afford DHFR-CO-CH_2-S-CO-CH_3. The acetylthioacetylated DHFR is deprotected by the addition of 25 uL of a pH 7.5 solution containing 100 mM sodium phosphate, 25 mM EDTA, 100mM NH_2OH. The reaction proceeds for two hours at room temperature after which the material is again passed down a PD-10 column by elution with PBS. The final product, DHFR-CO-CH_2-SH is used immediately.

(7b) Preparation of mercaptoalkyl-DHFR

A sample of DHFR (50 nmoles) is dissolved in 0.1 M carbonate buffer (pH 9) and 2 mmoles of an aqueous solution of 2-iminothiolane are added. The reactants are vortex mixed and kept at room temperature for 120 minutes. The reaction mixture is quenched by the addition of 2 mmoles of ethanolamine, diluted with phosphate buffed saline. The reaction mixture is added to a prewashed PD-10 column, and eluted with PBS to afford DHFR-HC(NH_2^+)$CH_2CH_2CH_2$SH. For use in conjugation to maleimide-deriviatitzed ING-1 (Example 6a), the product is eluted off the column directly into the antibody solution.

(7c) Preparation of reduced DHFR using dithiothreitol

A solution containing 40 nmoles of DHFR in PBS was vortexed and an equal volume of 500 mM dithiothreitol in PBS was added. After mixing and standing on ice for 60 minutes, the reaction mixture was eluted from a prewashed PD-10 column with PBS to afford DHFR-SH. For use in conjugation to maleimide-deriviatitzed antibody (Example 6a), the product was eluted off the column directly into the antibody solution.

(7d) Preparation of DHFR-Maleimide using Sulfo-SMCC

A sulfo-SMCC solution (300 nmoles) in PBS is added to a sample of DHFR (50 nmoles) in phosphate buffer (pH7). The resulting mixture is allowed to stand for 30 minutes with occasional mixing at room temperature. The reaction is stopped with 60 nmoles basic tris buffer. The reaction mixture is diluted with phosphate buffed saline, added to a prewashed PD-10 column, and eluted

with PBS to afford DHFR-maleimide. This material is stored on ice until use.

(7e) Radiolabeling of DHFR with ^{125}I

An aliquot of DHFR (500 ug) is labeled with ^{125}I monochloride (at about 5 mCi/mg) in the presence of iodogen (Sodium N-chloro-benzenesulfonamide) beads in a volume of 500 uL 100mm phosphate buffer (pH 7.2) at room temperature. After 15 minutes the reaction is terminated by passage of the labeled protein down a prewashed NAP-5 column. The iodinated DHFR is eluted with PBS and stored at 4°C until use.

(7f) Alternative conjugation method that protects DHFR active site

In order to prevent the interaction of reagents with the active site of the DHFR enzyme during the derivatization process, the active site of the enzyme is blocked to prevent the entry of the reagents.

A methotrexate/agarose (Sigma) column resin slurry (1 mL: protected from light) is washed twice with a large volume of high salt buffer (100 mM KPO_4, 1.0 M KCl, 1.0 mM K_2EDTA and 0.5 mM dithioerythritol at pH 6.0) to remove any free methotrexate. The final centrifuged pellet is mixed with DHFR (1 mmole/5 mL) in 5.0 mL of buffer containing (50 mM KPO_4, 1.0 mM K_2EDTA and 0.5 mM dithioerythritol at pH 6.0) and left for one hour. The resin with the now attached DHFR is centrifuged to a pellet and washed x3 with washing buffer (50 mM KPO_4, 1.0 mM K_2EDTA at pH 6.0). The resin pellet is suspended in a sulfo-SMCC solution (6 mmoles) in PBS (pH 7). The resulting mixture is stirred very slowly for 60 minutes at room temperature. The reaction is stopped by dilution with 10 mL of washing buffer and

the resin is again centrifuged to a pellet and washed 2x with the washing buffer. The resin is poured into a narrow glass Pasteur pipette whose exit is obstructed with a plug of glass wool. Maleimide derivatized DHFR is removed from the resin by elution with 30 mL of eluting buffer (100 mM folic acid, 200 mM KBO_3, 1.0 M KCl, 1.0 mM K_2EDTA at pH 9.0) to afford DHFR-maleimide. This material is pooled, dialysed against dialysis buffer (20 mM Tris, 1.0 mM K_2EDTA; pH 7.2) at 4°C overnight and then concentrated in a Centricon-10® (Amicon) device to a concentration of approximately 1.0 mg/mL protein. This material is then reacted with antibody containing a free sulfhydryl group.

The folate is removed from the active site of the enzyme by passage of the maleimide DHFR (Example 7a) or antibody-DHFR conjugate (see Example 8 below) through a DEAE-SEPHACEL column (Pharmacia). The column (~50 mL resin) is prewashed with DEAE-washing buffer (10 mM Tris, 1.0 mM K_2EDTA, 0.2 mM dithioerythritol; pH 7.2) and the protein loaded on in the same buffer. After washing, the protein is removed from the column with a non-linear gradient of 1000mL of DEAE-washing buffer (10 mM Tris, 1.0 mM K_2EDTA, 0.2 mM dithioerythritol; pH 7.2) to 1000 mL of 10 mM Tris, 0.5 M KCl, 1.0 mM K_2EDTA, 0.2 mM dithioerythritol; pH 7.2. Fractions eluting from the colum are collected and monitored at 280 nm for protein content. Fractions containing protein are pooled, concentrated as before and dialysed overnight against PBS to produce folate-free material.

Example 8

(8a) Conjugation of DHFR to antibody (formation of Z-L_1-Rec)

The methodologies for conjugation are essentially the same, irrespective of whether the maleimide group is on the antibody or on the DHFR, and irrespective of the method chosen to introduce the sulfhydryl group into the protein. The final molar ratio during the conjugation is maintained at close to equimolar antibody:DHFR in order to control over-conjugation of the proteins which could result in inactivation of one or other or both. The following procedure is applicable to the conjugation of materials in Example 6 to the materials in Example 7.

Following reduction (see Example 7a), a sample (50 nmoles) of DHFR(N)-CO-CH_2-SH is eluted off a PD-10 column directly into a solution of maleimide-deriviatitzed ING-1 (5 nmoles) prepared according to Example 6a. After a brief mixing the solution is rapidly concentrated by centrifugation in a Centricon-10® device to a concentration of approximately 3.0 mg/mL protein. The reaction then is allowed to proceed for 4 hours at room temperature. The antibody-DHFR conjugate is transfered to a Centricon-30® concentrator and diluted with PBS. After concentrating the protein down to a volume of approximately 500 uL by centrifugation, the material is again diluted with PBS to 3.0 mL and recentrifuged. This procedure, which separates unconjugated DHFR and other low molecular weight products from the retained antibody-DHFR and unconjugated antibody products, is repeated 4 times or until spectrophotometric monitoring of the filtrate at 280 nm shows that no further protein is being filtered. Finally, the material in the Centricon-30® is concentrated to approximately 1.0 mg, ING-1/DHFR, per

milliliter solution and applied to a 2.6 x 60 cm Sephacryl S-200 size-exclusion column equilibrated and eluted with 50 mM sodium phosphate buffer at pH 7.2 supplemented with 150 mM sodium chloride. This column separates unconjugated antibody from antibody-DHFR conjugate. Fractions of the eluate containing the conjugate are pooled and then centrifuged in a Centricon-30 device to a concentration of approximately 1.0 mg, ING-1/DHFR, per milliliter solution. The conjugate is sterile filtered through a 0.22 µ filter and stored at 4°C until use.

Addition of trace amounts of either ^{125}I-labeled DHFR or ^{125}I-labeled ING-1 to the reaction mixtures, allows the ratio of one protein to the other after conjugation to be calculated.

(8b) Conjugation of reduced DHFR to Antibody-malemide (Scheme 1)

Following reduction, a sample (50 nmoles) of DHFR-SH (Example 7c) was eluted off a PD-10 column directly into a solution of maleimide-deriviatitzed ING-1 (5 nmoles) prepared according to Example 6a. After a brief mixing, the reaction was allowed to proceed for 4 hours at room temperature. The antibody-DHFR conjugate was transfered to a Centricon-30® concentrator, diluted with PBS, and concentrated down to a volume of approximately 500 µL by centrifugation. The concentrated protein was then diluted with PBS to a volume of 3.0 mL and recentrifuged to a volume of 500 uL. This procedure, which separates unconjugated DHFR and other low molecular weight products from the retained antibody-DHFR and unconjugated antibody products, was repeated three times. Finally, the material in the Centricon-30® was concentrated to approximately 1.0 mg ING-1-DHFR per milliliter solution and applied to a 10 X 300 mm

Superose 12 FPLC size-exclusion column equilibrated and eluted at 1 mL/min with 50 mM sodium phosphate buffer (pH 7.2) supplemented with 150 mM sodium chloride.The optical density of the eluate was continuously monitored at 280 nm and fractions (1.0 mL) containing the antibody-DHFR conjugate were pooled. The protein concentration of the pooled material was assayed and the conjugate was stored at 4°C until use.

Examples of System B

SYSTEM B

	Non-Radioactive Targeting ImmunoReagent NRTIR	Radioactive Delivery Agent RDA
1	Immunoreactive group + (linking group + ligand)$_n$	Receptor + (linking group + chelating agent + radionuclide)$_m$
2	Z-(L_1-DHFR ligand)$_n$	Rec-(L_2-Q-M)$_m$
3	Z-(L_1-TMP)$_n$	Rec-(L_2-Q-M)$_m$
4	Z-(L_1-MTX)$_n$	Rec-(L_2-Q-M)$_m$

wherein:

Z is the residue of an immunoreactive group;

Rec is the residue of a receptor, preferably a DHFR receptor;

D is the residue of a ligand that has an affinity for non-covalent binding to the receptor, preferably to a DHFR receptor;

DHFR ligand is the residue of a ligand that has an affinity for non-covalent binding to a DHFR active site;

TMP is the residue of a trimethoprim analog;

MTX is the residue of a methotrexate analog;

L_1 and L_2 are each independently the residue of a linking group that may independently contain spacing group;

Q is the residue of a chelating group;

M is a radionuclide; and

n and m are each independently an integer greater than zero.

Example 9

(9a) Preparation of Antibody-Malemide with Sulfo-SMCC (ING-1-Maleimide

This product is prepared according to the procedure of Example 6a.

(9b) Preparation of trimethoprim-4'-O-acetic acid cysteine

To a mixture of resin containing L-Cys(COO-trityl) (30 mM: Advanced Chem Tech) and DCC in 150 ml of methylene chloride is added in portions a solution of 5-(4'-O-acetic acid) (30 mM) in 100 ml of methylene chloride. The resulting reaction mixture is stirred 2 days, and the solvent and the urea are removed to afford the blocked trimethoprim-4'-O-acetic acid cysteine, which upon mild hydrolysis with trifluoroacetic acid, yields trimethoprim-4'-O-acetic acid cysteine.

(9c) Conjugation of trimethoprim-4'-O-acetic acid cysteine to Antibody (formation of $Z-L_1-REC$)

Trimethoprim-4'-O-acetic acid cysteine (50 nmoles) prepared according to Example 9b is added directly into a solution of maleimide-derivatized ING-1 (5 nmoles) prepared according to Example 9a. After a brief mixing the solution is then allowed to proceed for 4 hours at room temperature with intermittent stirring. The antibody-trimethoprim conjugate is transferred to a Centricom-30® concentrator and diluted with PBS. After concentrating the protein down to a volume of approximately 500 µL by centrifugation, the material is again diluted with PBS to 3.0 mL and recentrifuged. This procedure, which separates unconjugated

trimethoprim and other lower molecular weight products from the retained antibody-trimethoprim and unconjugated antibody products, is repeated 4 times. Finally, the material in the Centricon-30® is concentrated to approximately 10 mg ING-1-trimethoprim per milliliter solution and applied to a 2.6 x 60 cm Sephacryl S-200 size-exclusion column equilibrated and eluted with 50 mM sodium phosphate buffer at pH 7.2 supplemented with 150 mM sodium chloride. This column separates unconjugated antibody from antibody-trimethoprim conjugate. Fractions of the eluate containing the conjugate are pooled and then centrifuged in a Centricon-30® device to a concentration of approximately 1.0 mg ING-1-trimethoprim per milliliter solution. The conjugate is sterile filtered through a 0.22 filter and stored at 4°C until use.

Example 10

(10a) Conjugation of DHFR to TMT (formation of $REC-L_2-Q$)

Terpyridine methylenetetraacetic acid (TMT) or a suitable derivative thereof can be conjugated to a protein molecule (DHFR) to yield a protein-TMT conjugate. The DHFR referred to below is of either bacterial origin, produced as a recombinant product from the cloned DHFR gene which is overexpressed in E.coli, or of human origin available as a recombinant protein.

DHFR (50 nmoles) is allowed to react with TMT-isothiocyanate (250 nmoles in 1.0 M carbonate, 150 mM sodium chloride buffer, pH 9.3 in an acid washed, conical, glass reaction vial. The solution is stirred briefly to mix the reactants and then left in the dark at room temperature. After 16 hours, the DHFR/TMT conjugate is separated from unconjugated TMT, by applying the reaction mixture to a PD-10 chromatography column, which has been pre-washed and equilibrated with

50 mM sodium acetate buffer containing 150 mM sodium chloride at pH 5.6. The pure conjugate is eluted off the column with 2.5 mL of that same buffer, and concentrated on a Centricon-10® concentration device.

(10b) <u>Radiolabeling of DHFR/TMT with ^{90}Y: (formation of Rec-(L$_2$-O-M)m)</u>

A volume of radioactive Yttrium chloride (^{90}Y in 0.04 M hydrochloric acid at a specific activity of >500 Ci/g: Amersham-Mediphysics) is neutralized using two volumes of 0.5 M sodium acetate pH 6.0. The neutralized ^{90}Y (1.0 mCi) is added to 1.0 mL of DHFR/TMT (1 mg/mL) in 50 mM sodium acetate buffer containing 150 mM sodium chloride at pH 5.6. The labeling is allowed to proceed for one hour and then the reaction mixture is loaded on to a PD-10 chromatography column which has been pre-washed and equilibrated in a buffer contaiing 50 mM sodium phosphate with 150 mM sodiumchloride pH 7.4 (PBS). The sample is eluted from the column with 1.5 mL of PBS. Fractions of radiolabeled DHFR/TMT (0.5 mL) are collected, assayed for radioactivity, and pooled. The labeling efficiency is determined by removing 1.0 uL of the sample and spotting it on to a Gelman ITLC-SG strip. The strip is developed in a glass beaker contining 0.1 M sodium citrate, pH 6.0 for a few minutes until the solvent front has reached three-quarters of the way to the top of the paper. The strip is inserted into a System 200 Imaging Scanner (Bioscan) which has been optimized for ^{90}Y and is controlled by a Compaq 386/20e computer. In this system free ^{90}Y migrates at the solvent front while the DHFR/TMT (^{90}Y) remains at the origin. Using this system more than 98% of the total ^{90}Y radioactivity is found associated with DHFR/TMT at the origin.

Example 11

<u>Assays on the DHFR conjugates prepared from System A or System B</u>

(11a) <u>Protein Concentration</u>

The concentrations of ING-1 and DHFR for use in the conjugate reactions are determined by the BioRad protein assay (BioRad Catalog # 500-0001) using bovine immunoglobulin as the protein standard. By inclusion of trace amounts of ^{125}I-labeled DHFR or ING-1 in the reaction mixtures, and by knowing the specific activity of the preparations, the ratio of one protein to the other after conjugation is calculated.

As an alternative to radiolabelling, the DHFR can be tagged with other materials (e.g., TMT (for use with ^{90}Y or Europium fluorescence), biotin, fluorescene isothiocyanate (FITC) etc. to detect and quantify the amount of DHFR present in a solution or conjugated to another protein.

(11b) <u>Immunoreactivity assay by Flow Cytometry</u>

Conjugates of antibody-DHFR (e.g., Example 8) or antibody-trimethoprim (e.g., Example 10) are examined for their ability to bind to antigens on the surface of a human tumor cell line to which the antibody had been raised. The immunoreactivity of the conjugates is compared by flow cytometry with a standard preparation of the antibody before being subjected to modification. Target HT-29 cells (a human adenocarcinoma cell line obtained from the American Type Tissue Collection: ATTC) are grown to confluency in tissue culture flasks using McCoy's media supplemented with 10% fetal calf serum. The cells are harvested by scraping the flask walls with a cell scraper. Cells from many separate flasks are pooled, centrifuged to a pellet, resuspended at $5x10^5$/mL

in a solution of ice-cold 50 mM sodium phosphate with 150 mM sodium chloride buffer pH 7.4 (PBS) supplemented with 0.1% bovine serum albumin (Sigma) and 0.02% sodium azide (Flow buffer). The cells are washed in this same buffer and then counted. An antibody standard curve is constructed by diluting a stock solution of ING-1 with an irrelevant (non-binding), isotype-matched control antibody (human IgG_1), to give a number of samples ranging in ING-1 content from 10% to 100%. The standard curve is made in flow buffer so that each sample contains 1.0 mg protein per mL. Samples from the standard curve and ING-1-DHFR unknowns, or ING-1-trimethoprim unknowns are then incubated with $5x10^5$ HT29 cells at 4°C for 1 hour. After extensive washing to remove unbound antibody, the cells are resuspended in 100 uL flow buffer and incubated at 4°C for 1 hour with goat-anti-human antibody labelled with fluorescene isothiocyanate. After further washing in flow buffer the samples are analyzed by flow cytometry on a Coulter EPICS 753 flow cytometer. Fluorescein isothiocyanate (FITC) and propidium iodide (PI) are excited using the 488 nm emission line of an argon laser. The output is set at 500 mw in light regulation mode. Single cells are identified by 90 degree and forward angle light scatter. Analysis windows are applied to these parameters to separate single cells from aggregates and cell debris. Fluorescence from FITC and propidium are separated with a 550 nm long pass dichroic filter and collected through a 530 nm band pass filter (for FITC), and a 635 nm band pass filter (for PI). Light scatter parameters are collected as integrated pulses and fluorescence is collected as log integrated pulses. Dead cells are excluded from the assay by placing an analysis window on cells negative for PI uptake. The mean fluorescence per sample (weighted average from 2500 cells) is calculated for each histogram. FITC calibration beads are analysed

in each experiment to establish a fluorescence standard curve. The average fluorescence intensity for each sample is then expressed as the average FITC equivalents per cell. Immunoreactivity is calculated by comparing the average fluorescence intensity of the ING1-DHFR or ING-1-trimethoprim sample with values from the standard curve.

(11c) Immunoreactivity assay by ELISA

The antigen to which the antibody, ING-1, binds is prepared from LS174T or HT-29 cells (available from ATTC) by scraping confluent monolayers of cells from the walls of culture flasks with a cell scraper. The cells from many flasks are combined and a sample is taken and counted to estimate the total number of cells harvested. At all times the cells are kept on ice. Following centrifugation of the cells at 1500 rpm for 10 minutes at 4°C, the cells are washed once in 25 mL ice-cold 50 mM sodium phosphate buffer, pH 7.4 supplemented with 150 mM sodium chloride (PBS), pelleted under the same conditions and transfered in 10 mL PBS to an ice-cold glass mortar. The cells are homogenized at 4°C using a motor-driven pestle and then centrifuged at 3000 x g for 5 minutes. The antigen-rich supernatant is removed from the other cell debris and subjected to further centrifugation at 100,000 x g for one hour at 4°C. The pellet (antigen fraction) from this final step is suspended in 100 µL of PBS for every million cells harvested. Following an estimate of the protein concentration (BioRad BCA protein assay using bovine immunoglobulin as the protein standard) the antigen is stored at -20°C until use.

Each well of a 96-well Costar microtiter plates is coated with antigen by adding 100 µL/well of cell lysate (10 mg/ml) prepared as above. The microtitre plates are

allowed to dry overnight in a 37°C incubator. After washing the plate five times with 0.05% Tween-20 (Sigma), they were blotted dry. The wells of each plate were blocked by adding 125 µL/well of a 1% BSA (bovine serum albumin, Sigma) solution in PBS and incubated for 1 hour at room temperature. The plates were washed five times with 0.05% Tween-20. Samples (50 µL/well in duplicate) of ING-DHFR or ING-trimethoprim conjugates and standard ING-1 antibody solutions are prepared at a range of concentrations in 1% BSA in PBS. Biotinylated ING-1 (1.0 mg/mL in 0.1% BSA) is added to each well (50 µL/well) and the plates are then incubated for 2 hours at room temperature. Following five washes with 0.05% Tween-20, the plates are blotted dry and incubated at room temperature for one hour with dilute (1:2000 in 0.1% BSA) streptavidin-alkaline phosphatase (Tago). After a further five washes, color is developed in each well upon the addition of 100 µL per well of phosphatase substrate reagent (Sigma). After one hour at room temperature, the color wis read using a 405 nm filter in a Titertek Multiscan microplate reader.

(11d) <u>SDS PAGE gel electrophoresis</u>

Samples of ING-1-DHFR or ING-1-trimethoprim conjugates are subjected to electrophoresis on Novex 8%-16% reduced and native polyacrylamide gels using SDS buffers to estimate their apparent molecular weight and the degree of heterogeneity of the preparation. Using standards of known molecular weight run on the same gel, a standard curve is constructed of the distance travelled versus the log of the molecular weight. From this standard curve the relative molecular weights of the bands associated with each conjugate preparation are determined.

(11e) <u>Determination of aggregate formation by size-exclusion HPLC</u>

A 30 cm x 7.5 mm TSK-G3000SW size-exclusion HPLC column (Supelco) fitted with a guard column of the same material is equilibrated with 12 column volumes of 10 mM sodium phosphate buffer pH 6.0 supplemented with 150 mM sodium chloride using a Waters 600E HPLC system with a flow rate of 1.0 mL per minute at 400-600 PSI. A sample (25 uL) of BioRad gel filtration protein standards is injected on to the column. The retention time of each standard is monitored by a Waters 490 UV detector set at 280 nm. Following the recovery of the final standard, the column is washed with a further 10 volumes of 10 mM sodium phosphate buffer pH 6.0 supplemented with 150 mM sodium chloride. Samples (50 µL) of either native ING-1 antibody or ING-1-DHFR at 200 µg/mL are injected on to the column and their retention times recorded. From the areas of the retained peaks and the retention time, the amount of aggregated material in the ING-1-DHFR or ING-1-trimethoprim conjugates sampled are calculated.

(11f) <u>Determination of DHFR activity</u>

Among other things, the enzymatic activity of DHFR is used to monitor:

i) Preservation of enzyme activity in a manner analogous to measuring the binding of the antibody to it's antigen, the activity of the enzyme before and after conjugation is assayed to ensure that the act of conjugation does not inhibit the enzyme;

ii) inhibitory effect of drugs (e.g., trimethoprim and the trimethoprim analogs described above in Examples 1-5) on both free and antibody-conjugated DHFR;

iii) to assay the effect of the trimethoprim-based TMT [^{90}Y] delivery system; and

iv) measurement of the amount of DHFR in a solution.

According to the 1992 Sigma Chemical Company catalog on page 350, DHFR activity is defined in terms of units; one said unit of DHFR enzyme activity is defined as the amount of material needed to convert 1.0 micromole of 7,8-dihyrofolic acid and the reduced form of β-nicotinamide adenine dinucleotide phosphate (NADPH) to 5,6,7,8-tetrahydrofolate and the oxidized form of β-nicotinamide adenine dinucleotide phosphate (NADP) per minute at pH 6.5 and 25°C)

The activity of the enzyme was measured spectrophotometrically at 340 nm by following the the rate of oxidation of NADPH to NADP during the reduction of dihydrofolate to tetrahydrofolate. An aliquot (362 µL) of a freshly thawed solution of the enzyme substrate (7,8, dihyrofolic acid (5.44 mM)) was treated with 38 µL β-mercaptoethanol and 600 µL of 100mM Imidazole buffer pH 7.0. To measure the enzyme activity of DHFR, the reaction mixture contained 20 µl (3.2 mg/mL) of NADPH, 20 µL of the above 7,8, dihyrofolic acid mixture, and 955 µL 100mM Imidazole buffer pH 7.0. A sample (5 µL; 120 ng of protein) of DHFR was added to the prewarmed (25°C) reaction mixture, mixed rapidly, and the change in optical density at 340 nm was monitored every 5 seconds for a total of 120 seconds in a Shimadzu 160U ultraviolet spectrophotometer. The activity (units/mL) was calculated from the slope of the optical density vs time plot.

(11g) Inhibition of DHFR activity

Trimethoprim (TMP) or the trimethoprim-4'-O-acetic acid amide of H_2N-[D-Glu]-Ala-Ala-Ala-Ala-Lys-Lys-OH (TMP-peptide: Example 3), in a range of concentrations, was preincubated with samples of DHFR or ING-1-DHFR

conjugates (Example 8b) for 2 minutes. The activity of DHFR was then assayed as before (Example 11f) and the concentration of TMP or TMP-peptide required to produce 50% inhibition of enzyme activity (IC_{50}) was calculated.

Inhibitor	DHFR alone IC_{50} Concentration	ING-1/DHFR Conjugate IC_{50} Concentration
TMP	< 1.0 nMolar	1.0 nMolar
TMP-Peptide	4.2 nMolar	1.0 nMolar

(11h) Quantitation of DHFR in ING-1-DHFR conjugates.

A sample (1.2 µg of protein) of ING-1/DHFR conjugate (from Example 8b) was used in place of DHFR in the assay for enzyme activity (Example 11f) . The activity of the conjugate (units/mL) was calculated from the slope of the optical density vs time plot as before. From the activity of the conjugate prepared in Example 8b and the activity of a known standard amount of unconjugated DHFR, the average number of molecules of DHFR conjugated to each antibody molecule was calculated and found to be 0.34 moles of DHFR per mole of antibody.

The present invention has been described in detail with particular reference to certain preferred embodiments thereof, but it will be understood that variations and modifications can be effected within the spirit and scope of the invention.

We claim:

1. A non-radioactive targeting immunoreagent that comprises an immunoreactive material, the residue of one or more receptor moieties or ligands which have an affinity for non-covalent binding to a receptor moiety, and one or more linking groups.

2. A radioactive targeting immunoreagent that comprises the residue of a receptor moiety or a ligand which has an affinity for non-covalent binding to a receptor moiety, one or more chelating agents, one or more linking groups and one or more radionuclides.

3. A targeting immune reagent that comprises moieties represented by the structure

$Z-(L_1-X)_n$

wherein:

Z comprises the residue of an immunoreactive protein;

L_1 is a chemical bond or a linking group that may contain a spacing group;

X is the residue of a receptor moiety or a ligand which has an affinity for non-covalent binding to a receptor moiety; and

n is an integer greater than zero.

4. A radioactive targeting reagent comprised of moieties represented in the structure

$D-(L_2-Q-M)_m$

wherein:

D is the residue of a receptor moiety or a ligand which has an affinity for non-covalent binding to a receptor moiety;

L_2 is a chemical bond or a linking group that may contain a spacing group;

Q is the residue of a chelating group;

M is a radionuclide; and

m is an integer greater than zero.

5. The reagent of claim 3 wherein Z is the residue of an antibody or antibody fragment.

6. The reagent of claim 5 wherein the antibody is selected from ING-1; B72.3; 9.2.27; D612; UJ13A; NRLU-10; 7E11C5; CC49; TNT; PR1A3; B174; B43; and anti-HLB antibodies.

7. The reagent of claim 3 wherein X is the residue of dihydrofolate reductase.

8. The reagent of claim 7 wherein the residue of dihydrofolate reductase is derived from Escherichia coli strain CV634 transformed with the plasmid pCV29.

9. The reagent of claim 3 wherein X is selected from the group consisting of residues of trimethoprim analogues and residues of methotrexate analogs.

10. The reagent of claim 3 wherein L_1 is comprised of the residue of a heterobifunctional cross-linking reagent.

11. The reagent of claim 10 wherein the heterobifunctional cross-linking reagent is selected from the group consisting of sulfosuccinimidyl 4-(N-maleimidomethyl)cyclohexane-1-carboxylate, sulfosuccinimidyl (4-iodoacetyl)aminobenzoate, sulfosuccinimidyl 4-(p-maleimidophenyl)butyrate, 2-Iminothiolane, and N-succinimidyl S-acetylthioacetate.

12. The reagent of claim 3 wherein L_1 is comprised of the residue of a modified receptor moiety containing a reactive functional group.

13. The reagent of claim 12 wherein the reactive functional group is selected from the group consisting of amino groups and sulfhydryl groups.

14. The reagent of claim 4 wherein D is selected from the group consisting of residues of trimethoprim analogues and residues of methotrexate analogs.

15. The reagent of claim 4 wherein D is the residue of dihydrofolate reductase.

16. The reagent of claim 15 wherein the residue of dihydrofolate reductase is derived from Escherichia coli strain CV634 transformed with the plasmid pCV29.

17. The reagent of claim 4 wherein L_2 is the residue of a heterobifunctional cross-linking reagent.

18. The reagent of claim 17 wherein the heterobifunctional cross-linking reagent is selected from the group consisting of sulfosuccinimidyl 4-(N-maleimidomethyl)cyclohexane-1-carboxylate, sulfosuccinimidyl (4-iodoacetyl)aminobenzoate, sulfosuccinimidyl 4-(p-maleimidophenyl)butyrate, 2-Iminothiolane, and N-succinimidyl S-acetylthioacetate.

19. The reagent of claim 4 wherein L_2 is the residue of a modified ligand moiety containing a reactive functional group.

20. The reagent of claim 19 wherein the reactive functional group is selected from the group consisting of amino groups and sulfhydryl groups.

21. The reagent of claim 4 wherein Q contains a polycarboxylic acid group.

22. The reagent of claim 4 wherein Q is selected from the group consisting of B4A, P4A, TMT, DCDTPA, PheMT, macroPheMT, and macroTMT.

23. The reagent of claim 4 wherein M is a radioactive isotope of a metal.

24. The reagent of claim 23 wherein the radioactive isotope is selected from ^{212}Pb, ^{212}Bi, ^{90}Y, ^{177}Lu, ^{186}Re, ^{188}Re, ^{64}Cu, ^{67}Cu, ^{99m}Tc, ^{111}In, and ^{87}Y.

25. A method of making a compound of the structure:

$Z-(L_1-X)_n$

wherein:

Z comprises the residue of an immunoreactive protein;

L_1 is a chemical bond or a linking group that may contain a spacing group;

X is the residue of a receptor moiety or the residue of a ligand which has an affinity for non-covalent binding to a receptor moiety; and

n is an integer greater than zero;

comprising:

(i) reacting X with a precursor to a residue of L_1 under conditions and for a time period sufficient to form a covalent complex which is a precursor to a residue of L_1-X; and

(ii) reacting Z with the precursor to the residue of L_1-X produced in step (i) under conditions and for a time period sufficient to form a covalent complex Z-$(L_1$-X$)_n$.

26. The method of claim 25, wherein step (ii) comprises:
(iia) reacting Z with a precursor to the residue of L_1 under conditions and for a time period sufficient to form a covalent complex which is a precursor to a residue of Z-$(L_1)_n$; and
(iib) reacting the precursor to a residue of Z-$(L_1)_n$ produced in step (iia) with a precursor to a residue of L_1-X under conditions and for a time period sufficient to form a covalent complex Z-$(L_1$-X$)_n$.

27. A method of making a compound of the structure:
D-$(L_2$-Q-M$)_m$
wherein:
D is the residue of a receptor moiety or the residue of a ligand which has an affinity for non-covalent binding to a receptor moiety;
L_2 is a chemical bond or a linking group that may contain a spacing group;
Q is the residue of a chelating group;
M is a radionuclide; and
m is an integer greater than zero;
comprising:
(i) reacting Q with a precursor to a residue of L_2 under conditions and for a time period sufficient to form a covalent complex which is a precursor to a residue of L_2-Q
(ii) reacting D with the precursor to the residue of L_2-Q produced in step (i) under conditions and for a time period sufficient to form a covalent complex D-$(L_2$-Q$)_m$; and

(iii) reacting said covalent complex D-$(L_2$-Q$)_m$ with M under conditions and for a time period sufficient to form a complex D-$(L_2$-Q-M$)_m$.

28. The method of claim 27, wherein step (ii) comprises:
(iia) reacting D with a precursor to a residue of L_2 under conditions and for a time period sufficient to form a covalent complex which is a precursor to a residue of D-$(L_2)_m$; and
(iib) reacting the precursor to the residue of D-$(L_2)_m$ produced in step (iia) with a precursor to a residue of L_2-Q under conditions and for a time period sufficient to form a covalent complex D-$(L_2$-Q$)_m$.

29. The method of claim 25 wherein Z is an antibody or antibody fragment.

30. The antibody of claim 29 wherein the antibody is selected from ING-1; B72.3; 9.2.27; D612; UJ13A; NRLU-10; 7E11C5; CC49; TNT; PR1A3; B174; B43; and anti-HLB antibodies.

31. The method of claim 25 wherein X is selected from the group consisting of residues of trimethoprim analogs and residues of methotrexate analogs.

32. The method of claim 25 wherein L_1 is comprised of the residue of a heterobifunctional cross-linking reagent.

33. The method of claim 32 wherein the heterobifunctional cross-linking reagent is selected from the group consisting of sulfosuccinimidyl 4-(N-maleimidomethyl)cyclohexane-1-carboxylate, sulfosuccinimidyl (4-iodoacetyl)aminobenzoate,

sulfosuccinimidyl 4-(p-maleimidophenyl)butyrate, 2-Iminothiolane, and N-succinimidyl S-acetylthioacetate.

34. The method of claim 25 wherein L_1 is comprised of the residue of a modified nucleotide moiety containing a reactive functional group.

35. The method of claim 34 wherein the reactive functional group is selected from the group consisting of amine groups and sulfhydryl groups.

36. The method of claim 25 wherein X is the residue of dihydrofolate reductase.

37. The method of claim 36 wherein the residue of dihydrofolate reductase is derived from Escherichia coli strain CV634 transformed with the plasmid pCV29.

38. The method of claim 28 wherein D is selected from the group consisting of residues of trimethoprim analogs and residues of methotrexate analogs.

39. The method of claim 28 wherein D is the residue of dihydrofolate reductase.

40. The method of claim 39 wherein the residue of dihydrofolate reductase is derived from Escherichia coli strain CV634 transformed with the plasmid pCV29.

41. The method of claim 28 wherein L_2 is comprised of the residue of a heterobifunctional cross-linking reagent.

42. The method of claim 41 wherein the heterobifunctional cross-linking reagent is selected from the group consisting of sulfosuccinimidyl 4-(N-

maleimidomethyl)cyclohexane-1-carboxylate, sulfosuccinimidyl (4-iodoacetyl)aminobenzoate, sulfosuccinimidyl 4-(p-maleimidophenyl)butyrate, 2-Iminothiolane, and N-succinimidyl S-acetylthioacetate.

43. The method of claim 41 wherein L_2 is a residue of a ligand moiety containing a reactive functional group.

44. The method of claim 43 wherein the reactive functional group is selected from the group consisting of amine groups, carboxylate groups, hydroxyl groups, and sulfhydryl groups.

45. The method of claim 28 wherein Q contains a polycarboxylic acid group.

46. The method of claim 28 wherein Q is selected from the group consisting of B4A, P4A, TMT, DCDTPA, PheMT, macroPheMT, and macroTMT.

47. The method of claim 28 wherein M is a radioactive isotope of a metal.

48. The method of claim 47 wherein the radioactive isotope is selected from ^{212}Pb, ^{212}Bi, ^{90}Y, ^{177}Lu, ^{186}Re, ^{188}Re, ^{64}Cu, ^{67}Cu, ^{99m}Tc, ^{111}In, and ^{87}Y.

49. A pharmaceutical composition comprising a compound of claim 3 dissolved or dispersed in a pharmaceutically acceptable carrier.

50. A pharmaceutical composition comprising a compound of claim 4 dissolved or dispersed in a pharmaceutically acceptable medium.

51. A method of treating a tumor in a mammal comprising administering to said mammal an effective dose of a non-radioactive targeting immunoreagent of claim 3 in a pharmaceutically acceptable medium, waiting for a time period sufficient for said non-radioactive targeting immunoreagent to accumulate at the tumor site in said mammal, and subsequently, administering an effective dose of a radioactive targeting reagent of claim 4 in a pharmaceutically acceptable medium to said mammal, and waiting for a time period sufficient for said radioactive targeting reagent to accumulate at the target site, said target site being the said non-radioactive targeting immunoreagent accumulated at said tumor site in said mammal.

52. A method of diagnostic imaging in a mammal comprising administering to said mammal an imaging effective dose of a non-radioactive targeting immunoreagent of claim 3 in a pharmaceutically acceptable medium, waiting for a time period sufficient for said non-radioactive targeting immunoreagent to accumulate at the imaging site in said mammal, and subsequently, administering an effective dose of a radioactive targeting reagent of claim 4 in a pharmaceutically acceptable medium to said mammal, and waiting for a time period sufficient for said radioactive targeting reagent to accumulate at the target site, said target site being the said non-radioactive targeting immunoreagent accumulated at said imaging site in said mammal, and generating an image of said mammal.

53. The reagent of claim 3 wherein X is a residue of a receptor moiety and Z and X comprise a fusion protein.

54. The reagent of claim 53 wherein the receptor moiety is dihydrofolate reductase.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US93/11842

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) : A61K 49/02, 43/00; G01N 33/53
US CL :435/6, 7.1, 7.2, 7.23, 7.9, 26; 424/1.1, 9, 85.8
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/6, 7.1, 7.2, 7.23, 7.5, 7.9, 26; 424/1.1, 9, 85.8

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X, P	Cancer Research, Volume 53, issued 15 May 1993, G.A. Hawkins et al, "Delivery of Radionuclides to Pretargeted Monoclonal Antibodies Using Dihydrofolate reductase and Methotrexate in an Affinity system", pages 2368-2373, especially the Abstract.	1-54
X ---- Y	Journal of Nuclear Medicine, Volume 28, No. 8, issued August 1987, D.J. Hnatowich et al, "Investigations of Avidin and Biotin for Imaging Applications", pages 1294-1302, especially the Abstract, the lower right-hand column of page 1295, and the upper left-hand column of page 1296.	1-5, 12, 13, 19, 20, 23, 24, 52 ---------------- 6, 10, 11, 17, 18, 21, 22, 25-30, 32-35, 41-51, 53

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
30 MARCH 1994	APR 06 1994
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer Toni R. Scheiner Jill Warden for Telephone No. (703) 308-0196

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US93/11842

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X ---- Y	Journal of Controlled Release, Volume 11, No. 1-3, issued 1989, V.P. Torchilin et al., "Antibody-Linked Chelating Polymers for Immunoimaging <u>In Vivo</u> ", pages 297-303, especially the Abstract.	1-5, 12, 13, 19, 20, 23, 24, 52 ---------------- 6, 10, 11, 17, 18, 21, 22, 25-30, 32-35, 41-51, 53
X ---- Y	Cancer Research, Volume 51, issued 01 November 1991, G. Paganelli et al, "Three-Step Monoclonal Antibody Tumor Targeting in Carcinoembryonic Antigen-positive Patients", pages 5960-5966, especially the Abstract.	1-5, 12, 13, 19, 20, 23, 24, 52 ---------------- 6, 10, 11, 17, 18, 21, 22, 25-30, 32-35, 41-51, 53

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US93/11842

B. FIELDS SEARCHED

Electronic data bases consulted (Name of data base and where practicable terms used):

DIALOG: Medline, CAB Abstracts, Biosis, Embase, Cancerlit, Derwent; Automated Patent System
SEARCH TERMS: imaging, target?, sequential, pretarget?, post label?, dihydrofolate reductase, antibod?, radiolabel?, radioisotope, isotope, vivo, two step, three step, trimethoprim, methotrexate, Snow R A, Kruse L I, Black C D V, Shearman C W

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